The Role of *Hoxa2* Gene in Oligodendrocyte Development

A thesis submitted to the College of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, Saskatchewan

By

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Abstract

Although numerous transcription factors (TFs) are expressed by oligodendrocytes (OGs), the role(s) of most of these TFs in oligodendrogenesis remains to be elucidated. One such TF is Hoxa2, which was recently shown to be expressed by O4-positive ($^{+}$) pro-OGs. Hence, the main objectives of this thesis were to determine the expression profile and function(s) of Hoxa2 during OG development. Immunocytochemical analysis of primary mixed glial cultures demonstrated that Hoxa2 is expressed throughout oligodendrogenesis, diminishing only with the acquisition of a myelinating phenotype. Subsequently, immunohistochemical analysis suggested that Hoxa2 is expressed by migratory oligodendroglial cells in the embryonic spinal cord. However, double immunofluorescent analysis of *Hoxa2* transgenic knockout mice showed that OG specification and early maturation proceed normally in the absence of *Hoxa2* in the spinal cord.

As *Hoxa2* is one of 39 murine *Hox* genes, which exhibit extensive overlapping expression profiles in the spinal cord, we decided to examine the expression of an additional Hox TF, Hoxb4, during OG development. Immunocytochemical analysis of primary mixed glial cultures demonstrated that Hoxb4 is also expressed throughout OG development. Furthermore, comparison of the expression profiles of Hoxb4 and Olig2 suggested that Hoxb4 is expressed by oligodendroglial cells in the spinal cord. Hence, Hoxb4, as well as other Hox TFs could compensate for Hoxa2 in the spinal cord in its absence.

As the anterior boundary of most *Hox* genes has been found to be in the hindbrain or spinal cord, we decided to look at the telencephalon which would be less likely to have compensatory mechanisms. Our results showed that similar to the spinal cord, Hoxa2 is expressed by oligodendroglial cells in the telencephalon. Subsequently, it was found that over-expressing *Hoxa2* in CG4 cells, an oligodendroglial cell line derived from the perinatal rat cerebral cortex, impairs their differentiation. In an attempt to determine the mechanism by which it accomplishes this, we examined the expression of polysialylated neural cell adhesion molecule (PSA-NCAM), which has been implicated in this process. Contrary to our expectations, however, it was found that over-expressing *Hoxa2* in CG4 cells results in significantly fewer PSA-NCAM^{+} cells. Hence, the results

suggest that Hoxa2's effect on OG differentiation is independent of its effect on PSA-NCAM expression.

The expression of *Hox* genes is enhanced by retinoic acid (RA), which, in turn, both inhibits, as well as promotes OG differentiation. Although the reason for these opposing roles is uncertain, examination of the experimental protocols utilized by different research groups reveals disparities in age, CNS region, as well as RA concentration. As a result, RA's effect on oligodendrogenesis could be stage- and/or concentration-dependent. In order to determine which of these factors could impact RA's effect on OG differentiation we treated CG4 cells with two different concentrations of RA at two distinct time points. The results showed that both factors (concentration and time/stage) can impact RA's effect on CG4 cell differentiation. In an attempt to determine the mechanism by which it accomplishes this, we examined the expression of PSA-NCAM. Contrary to our expectations, the results suggest that RA's effect on CG4 differentiation is independent of its effect on PSA-NCAM expression. The results of this thesis suggest that Hoxa2 and RA could play multiple roles in OG development. Although these roles appear to be similar, further research will be needed to determine whether Hoxa2 acts a downstream effector in the RA signaling pathway in oligodendroglial cells.

Acknowledgements

I would like to thank:

- my supervisors Drs. Adil Nazarali and Ronald Doucette for their advice and support over the years.
- my advisory committee members (Drs. Ric Devon, Marianna Foldvari, Valerie Verge, Brian Bandy, and Jane Alcorn) for taking time out of their busy schedules to be on my committee.
- Dr. V. Wee Yong for agreeing to be the external advisor for my defense.
- my husband and parents for their support and love throughout my university studies.
- Ms. Sheng Yu and Mrs. LaRhonda Sobchishin for their technical assistance. (I would especially like to thank LaRhonda for her friendship over the past three years and for always having an open ear for frustrations and worries regarding my research.)
- the College of Pharmacy and Nutrition, the University of Saskatchewan, the College of Graduate Studies and Research, the Canadian Institute of Health Research, Rx&D, the Saskatchewan College of Pharmacists, the Saskatoon Division of the Canadian Federation of University Women, and the Association of Faculties of Pharmacy of Canada for their financial assistance throughout my university studies.

Publications

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Reviews

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List of Abbreviations

AEP	anterior entopenduncular area
ANOVA	analysis of variance
ATCC	American type cell culture
bHLH	basic helix-loop-helix
BMPs	bone morphogenetic proteins
Brn	Brain
BrdU	bromodeoxyuridine
C	Carnegie
$Ca_3(PO_4)_2$	Calcium phosphate
$CaCl_2$	Calcium chloride
CAT	chloramphenicol acetyltransferase
CBF1	C promoter binding factor 1
cDNA	complementary deoxyribonucleic acid
CG4	central glia 4
CGE	Caudal ganglionic eminence
CNP	2′,3′-cyclic nucleotide 3′-phosphodiesterase
CNS	central nervous system
CO_2	carbon dioxide
COUP-TFI	Chicken ovalbumin upstream promoter-transcription factor I
CRABPII	cellular retinoic acid binding protein II
CYP26	cytochrome P450, family 26
DAB	diaminobenzidine tetrahydrochloride
Dbx2	developing brain homeobox 2
DIV	day(s) *in vitro*
DMEM	Dulbecco's modified Eagle's medium
dp	dorsal progenitor domains
dpc	days postcoitum
dVZ	dorsal ventricular zone
E	embryonic age
EDTA	ethylenediamine-tetraacetic acid
EGL	external granule cell layer
Emx1	empty spiracles homolog 1
endoN	endoneuraminidase
E2F-1	E2F transcription factor 1
Evx1	even skipped homeotic gene 1 homolog
FBS	fetal bovine serum
FGF	Fibroblast growth factor
FITC	fluorescein isothiocyanate
FP	floor plate
GalC	galactocerebroside
GFAP	glial fibrillary acidic protein
Gli2	GLI-Krüppel family member Gli2
Gsh2	genomic screened homeobox 2
h	hour(s)

HBS	homeodomain binding sites
HCl	hydrochloric acid
HLH	helix-loop-helix
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
Hes5	Mammalian homolog of *hairy* and *Enhancer of split*
HOX/Hox	Homeobox of the Antennapedia class of genes (Human/rodent)
Id	Inhibitor of DNA binding
IgG	immunoglobulin G
IgM	immunoglobulin M
Irx	Iroquois
kD	kilodalton
Krox	Krüppel box
kV	kilovoltage
Lbx1	ladybird homeobox homolog 1
LGE	Lateral ganglionic eminence
LIF	leukemia inhibitory factor
M	molar
MAG	Myelin associated glycoprotein
Mad	*mothers against decapentaplegic*
Mash1	Mammalian *achaete-scute* homolog 1
MBP	Myelin basic protein
MGE	medial ganglionic eminence
min	minute(s)
ml	milliliter
mM	millimolar
mm	millimeter
MN(s)	motor neuron(s)
MOG	myelin oligodendrocyte glycoprotein
MOM	Mouse on Mouse
MS	Multiple sclerosis
MyTI	Myelin transcription factor I
N	normal
NCAM	neural cell adhesion molecule
NFIA	Nuclear factor I A
ng	nanogram
Ngn	Neurogenin
Nkx2.2	NK2 transcription factor related, locus 2
nM	nanomolar
NSCs	neural stem cells
OG(s)	Oligodendrocyte(s)
Olig	Oligodendrocyte transcription factor
OPC(s)	Oligodendrocyte precursor cell(s)
O2A	oligodendrocyte type 2 astrocyte
P	Postnatal day
Pax6	Paired box gene 6
PBS	phosphate buffered saline

Pbx	pre B-cell leukemia transcription factor
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDGFαR	platelet-derived growth factor alpha-receptor
pHi	intracellular pH
Phox2b	paired-like homeobox 2b
PLP	Proteolipid protein
pMN	motor neuron progenitor
pMNv	visceral motor neuron progenitor
POU	Pit Oct Unc
PP-MS	primary progressive MS
Prep/Meis	Pbx regulating protein gene/Myeloid ecotropic viral integration site
PSA-NCAM	polysialylated neural cell adhesion molecule
PVDF	polyvinylidene difluoride
r	rhombomere
RA	retinoic acid
Raldh	retinaldehyde dehydrogenases
RAR	Retinoic acid receptor
RARE	retinoic acid response element
rKr2	rat Krüppel-type protein 2
RP	roof plate
rpm	rotations per minute
RR-MS	relapsing-remitting MS
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
RXR	Retinoid X receptor
SCIP	Suppressed cAMP inducible POU
SDS	sodium dodecyl sulfate
Shh	sonic hedgehog
Hoxa2-S	Sense *Hoxa2*
siRNA	Short interfering RNA
SM	skim milk
Sma	*C. elegans* homolog of *Drosophila Schnurri*
Sox	SRY-box containing gene
T	Theiler
TAE	Tris acetate-EDTA
TAQ	*Thermus aquaticus*
Tet	tetracycline
TFs	transcription factors
tTA	tetracycline transcriptional activator
TX	Triton X
µF	microfarad
µg	microgram
µl	microliter
µM	micromolar
µm	micrometer

vVZ	ventral ventricular zone
WT	wild-type
Zfp488	Zinc finger protein for OG differentiation
$^{+}$	positive
$^{-}$	negative

Note: When word is italicized it is referring to the gene. When the word is not italicized it is referring to the protein (eg. *Hoxa2*: gene; Hoxa2: protein)

1.0 GENERAL INTRODUCTION

Oligodendrocytes (OGs) are the cells possessing the ability to assemble a myelin sheath around axons in the central nervous system (CNS) (Southwood et al. 2004). *In vitro* OG development progresses through a number of distinct stages characterized by specific antigenic phenotypes. In particular, during early stages of oligodendrogenesis, $A2B5^+$ oligodendrocyte precursor cells (OPCs) and $O4^+$ pro-OGs actively proliferate (Fok-Seang and Miller 1994) and migrate (Noble et al. 1988). Subsequently, loss of these traits and the emergence of galactocerebroside (GalC) expression (Fok-Seang and Miller 1994; Noble et al. 1988) signal terminal differentiation into pre-myelinating OGs. Progression to the mature myelinating phenotype occurs when the cells synthesize myelin proteins and elaborate sheet-like membranes (Duchala et al. 1995).

Oligodendroglial cells express a dynamic combination of transcription factors (TFs) as they progress from precursors to mature myelinating cells. Researchers have elucidated the roles of some of these TFs with the aid of transgenic knockout mice and/or transfection assays; however, the function(s) of most of these TFs remains to be determined. One such TF is Hoxa2, which is one of 39 mouse and human *Hox* genes that are organized into four clusters (*Hox* A, B, C, D) located on different chromosomes (Favier and Dollé 1997; Santagati and Rijli 2003; Scott 1992). These genes are characterized by a 180 base pair homeobox which encodes a 60 amino acid homeodomain (McGinnis et al. 1984a; McGinnis et al. 1984b). Through this homeodomain Hox TFs can regulate the expression of downstream effector genes by binding to specific nucleotide sequences (Akin and Nazarali 2005; Hirsch et al. 1990; Hoey and Levine 1988).

Hox gene expression, in turn, is enhanced by retinoic acid (RA), an active metabolite of vitamin A (Maden 2002). Interestingly, RA can either inhibit (Laeng et al. 1994; Noll and Miller 1994) or promote (Barres et al. 1994; Givogri et al. 2001; Pombo et al. 1999) OG differentiation. The reason for these opposing roles is uncertain; however, examination of the experimental protocols utilized by the individual research groups reveals differences in age, CNS region, as well as RA concentration. Therefore, RA's effect on oligodendrogenesis may be stage- and/or concentration-dependent.

How RA can inhibit or enhance OG differentiation is unknown; however, its inhibitory effect may be due to its ability to enhance the expression of polysialylated neural cell adhesion molecule (PSA-NCAM) (Husmann et al. 1989), a cell surface glycoprotein implicated in inhibiting OG differentiation (Decker et al. 2000). In particular, in carcinoma cell lines RA enhances the activity of sialyltransferase, which adds sialic acid residues to the NCAM protein, in a concentration-dependent manner (Deutsch and Lotan 1983). As this effect is first evident one day after RA treatment (Deutsch and Lotan 1983), downstream targets of RA, such as Hox TFs (Simeone et al. 1990), may be involved. Interestingly, several Hox TFs have been implicated in the regulation of NCAM expression (Jones et al. 1993; Jones et al. 1992). Therefore, RA may inhibit OG differentiation via enhancing the expression of PSA-NCAM, potentially with the aid of downstream effectors, such as Hox TFs.

Therefore, the aim of this thesis is threefold: to fully characterize Hoxa2 expression during OG development; to determine its role in this process, as well as its effect on PSA-NCAM expression; and, finally, to examine RA's effect on OG differentiation in relation to PSA-NCAM.

1.1 Objectives

1. To examine the expression of Hoxa2 during OG development.
2. To determine Hoxa2's role in oligodendrogenesis.
3. To examine the role of RA in OG development in relation to PSA-NCAM.

1.2 Hypotheses

1. Hoxa2 inhibits OG differentiation via enhancing the expression of PSA-NCAM.
2. RA inhibits OG differentiation via enhancing PSA-NCAM expression.

2.0 LITERATURE REVIEW

2.1 Introduction to oligodendrocyte development and its regulation

2.1.1 Specification

OGs are the myelinating cells of the CNS (Southwood et al. 2004). In the developing embryo, OPCs arise in multiple distinct foci located in various regions of the CNS. For example, in the developing murine spinal cord (Cai et al. 2005; Fogarty et al. 2005; Fu et al. 2002; Vallstedt et al. 2005), hindbrain (Vallstedt et al. 2005), and telencephalon (Kessaris et al. 2006) OPCs arise in both ventral and dorsal neuroepithelial domains [Figure 2.1; Table 2.1]. Subsequently, the OPCs must migrate out from these domains in order to populate specific CNS regions (Fogarty et al. 2005; Kessaris et al. 2006). Hence, the regulation of OG specification in these regions is critical for normal CNS functioning. As such, the purpose of this section of the literature review is to describe the regulation of OG specification, highlighting, when possible, any similarities or differences that exist in the ventral and/or dorsal regions of the spinal cord, hindbrain, and telencephalon.

2.1.1.1 Spinal cord

In the spinal cord, OPCs, which are characterized by platelet-derived growth factor alpha-receptor (PDGFαR) expression, initially arise in a distinct region of the embryonic ventral ventricular zone (vVZ) (Sun et al. 1998). This region is known as the motor neuron progenitor (pMN) domain [Figure 2.1A], since it gives rise to motor neurons (MNs) at earlier stages of embryonic development (Fu et al. 2002). The pMN domain, in turn, is one of five ventral progenitor domains formed in response to graded sonic hedgehog (Shh) concentrations (Briscoe et al. 2000; Gritli-Linde et al. 2001). In particular, Shh, which is expressed and secreted by cells of the notochord and floor plate (FP) (Echelard et al. 1993; Marti et al. 1995), initially regulates the expression of the TFs that define the boundaries of these progenitor domains (Briscoe et al. 2000; Ericson et al. 1997; Lu et al. 2000; Pierani et al. 1999). This includes regulating the expression of Nkx2.2 and Pax6 (Ericson et al. 1997), as well as *Olig2* (Lu et al. 2000) and *Irx3* (Briscoe et al. 2000), which define the ventral (Briscoe et al. 2000) and dorsal (Novitch et al. 2001) boundaries, respectively, of the pMN domain [Figure 2.2]. Subsequently, cross-repressive interactions between these TFs maintain and refine these boundaries

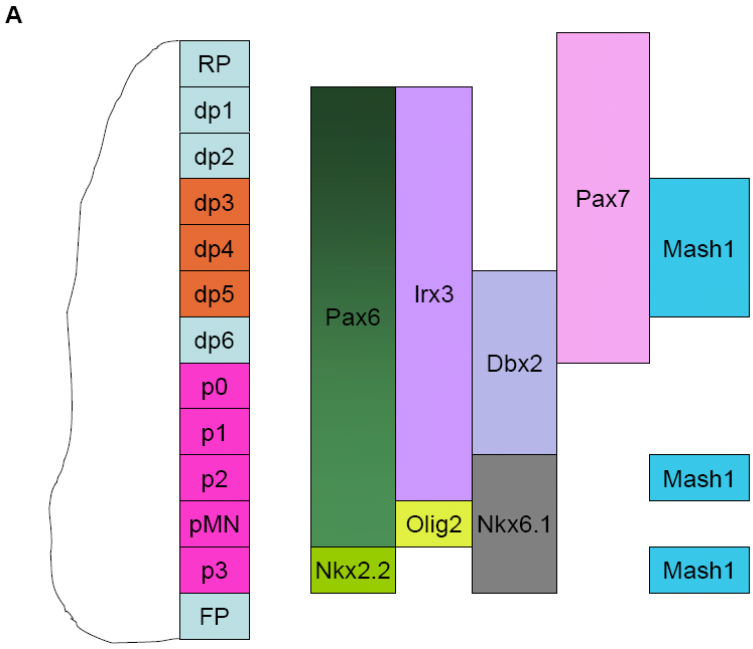
Figure 2.1 Oligodendrocyte precursor cells arise from both ventral (pink) and dorsal (orange) neuroepithelial domains in the spinal cord (A), hindbrain (B), and telencephalon (C).

(A) Illustration of a transverse section of the embryonic spinal cord showing the various progenitor domains, as well as the FP and roof plate (RP). PDGFαR^{+} OPCs initially arise from the pMN domain, which is specifically demarcated by the TF Olig2. Most of the information regarding the transcriptional control of OG specification pertains to this domain. However, OPCs also arise from other ventral progenitor domains in the rat, including p3 at embryonic age (E) 11.5 and p0-p2 at E16.5. In addition, OPCs also arise from three dorsal progenitor (dp) domains (shown in orange). (Some of the TFs involved in the dorsal-ventral patterning of the spinal cord are shown on the right.)

(B) Illustration of a transverse section of the embryonic hindbrain which shows where ventral (pink) and dorsal (orange) OPCs arise. To the right of the illustration are some TFs that are involved in the patterning of the ventral hindbrain. It is important to note that the domain from which ventral OPCs arise varies depending upon the anterior-posterior location, with OPCs arising from the visceral MN progenitor (pMNv) domain in the anterior hindbrain and the pMN domain in the posterior hindbrain. (The dorsal hindbrain is not shown due to lack of sufficient information regarding exactly where OPCs arise in this region.)

(C) Illustration of a coronal section of the embryonic telencephalon depicting where ventral (pink) and dorsal (orange) OPCs arise. The specific regions from which they arise are shown on the right along with some of the TFs known to pattern these regions.

Abbreviations: dVZ-dorsal ventricular zone; MGE-medial ganglionic eminence; LGE-lateral ganglionic eminence


A
RP
dp1
dp2
dp3
dp4
dp5
dp6
p0
p1
p2
pMN
p3
FP
Pax6
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Dbx2
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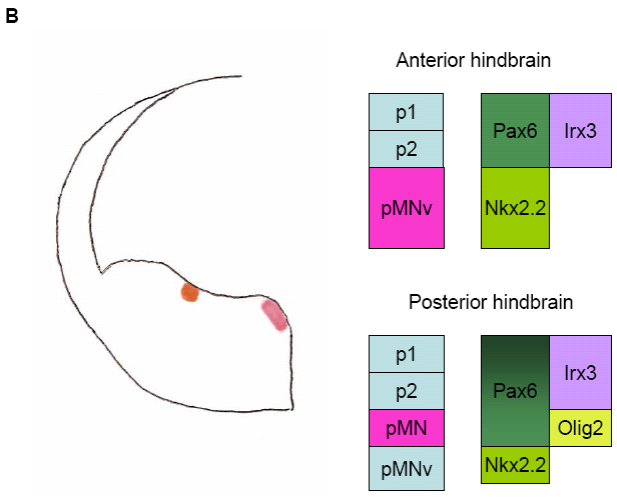
Nkx6.1
Mash1
Nkx2.2
Mash1


B
Anterior hindbrain
p1
p2
pMNv
Pax6
Irx3
Nkx2.2
Posterior hindbrain
p1
p2
pMN
pMNv
Pax6
Irx3
Olig2
Nkx2.2

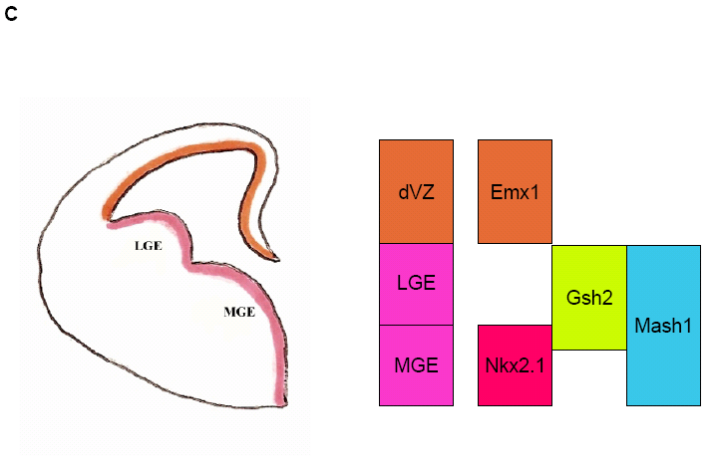

C
LGE
MGE
dVZ
Emx1
LGE
Gsh2
Mash1
MGE
Nkx2.1

Table 2.1 Oligodendrocyte precursor cells arise in ventral and dorsal regions of the spinal cord, hindbrain, and telencephalon.

CNS region	Ventral/ Dorsal	Age arise*	Region arise	Final destination	Reference
Spinal cord	Ventral	E11.5 (rat)	p3	Spinal cord	Sugimori et al. (2007)
		E12.5	pMN	Spinal cord	Fu et al. (2002); Lu et al. (2000)
		E16.5 (rat)	p0-p2	Spinal cord	Sugimori et al. (2007)
	Dorsal	~E15	dp3-5	Lateral funiculus (rare in the dorsal or ventral funiculi)	Cai et al. (2005); Fogarty et al. (2005); Vallstedt et al. (2005)
Hindbrain	Ventral	~E13.5	pMN,pMNv	Hindbrain	Vallstedt et al. (2005)
	Dorsal				
Telencephalon	Ventral	~E12.5	MGE/AEP	Preoptic area, corpus callosum, Anterior commissure, Lateral Olfactory Tract, Motor cortex	Ivanova et al. (2003); Kessaris et al. (2006)
		~E15	LGE/CGE		
	Dorsal	~P0	dVZ	Corpus callosum, Fimbria, Motor cortex	Gorski et al. (2002); Kessaris et al. (2006)

*listed as murine age unless otherwise stated

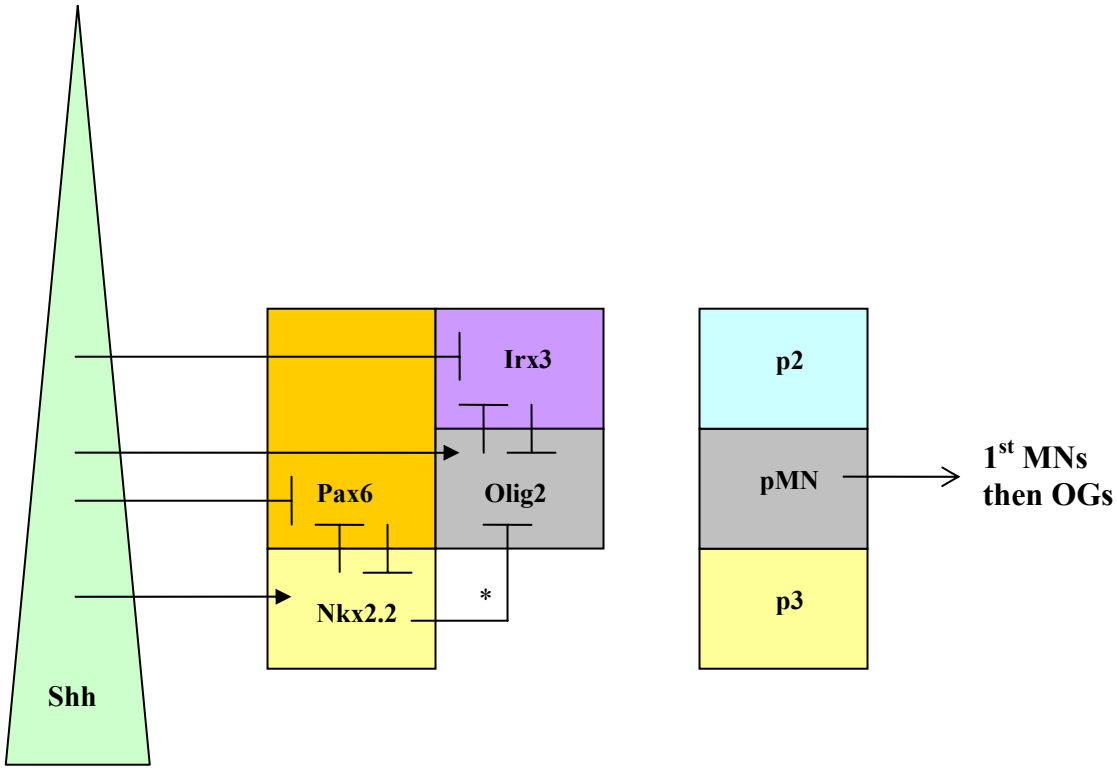


Figure 2.2 Sonic hedgehog signaling directs the formation of ventral progenitor domains, such as the pMN domain, which gives rise to motor neurons and subsequently oligodendrocytes.

Shh, which exhibits a ventral to dorsal decreasing expression gradient in the spinal cord, enhances (→) or represses (⊥) the expression of the TFs that define the boundaries of the pMN domain. These boundaries are further maintained and refined by repressive interactions between these TFs. (*: effect is only evident during early stages of development)

(Briscoe et al. 2000; Novitch et al. 2001).

Of the TFs that define the boundaries of the pMN domain only Olig2, which specifically demarcates this region (Fu et al. 2002; Novitch et al. 2001), is critical for OG specification in the ventral spinal cord. In fact, in its absence, the expression of *PDGFαR* is undetectable throughout the murine spinal cord (Lu et al. 2002). An additional Olig gene, *Olig1*, is also expressed in the pMN domain of rodents (Zhou et al. 2000); however, it is not critical for OG specification in this region since *PDGFαR* expression appears on schedule in its absence (Lu et al. 2002).

The function of *Irx3*, which defines the dorsal boundary of the pMN domain along with *Olig2* (Fu et al. 2002; Novitch et al. 2001), is currently unknown. However, it is likely to be important in spatial aspects of OG specification since it maintains and refines this boundary through cross-repressive interactions with Olig2 (Novitch et al. 2001). As such, it would be expected that in the absence of *Irx3* there would be a dorsal expansion of Olig2 expression and, therefore, OG specification. Furthermore, as early development appears to proceed from ventral to dorsal in the spinal cord (Altman and Bayer 1984), the predicted dorsal expansion of the $Olig2^{+}$ pMN domain could result in prolonged OG specification. Interestingly, recent research findings suggest that Irx3 may be indirectly enhanced by bone morphogenetic proteins (BMPs) (Meyer and Roelink 2003), which are dorsalizing signals expressed and secreted by the epidermal ectoderm and RP (Lee et al. 2000; Liem Jr. et al. 1997; Liem Jr. et al. 1995). This proposed Irx3 enhancement, in turn, may explain how BMPs are able to inhibit Olig2 expression and, hence, OG development in the ventral spinal cord (Mekki-Dauriac et al. 2002). Obviously, confirmation of these speculations will require further research.

Nkx2.2 and Pax6, which define the ventral boundary of the pMN domain (Briscoe et al. 2000), are important in temporal and/or spatial aspects of OG specification. In particular, Pax6-deficient mice exhibit a dorsal shift and a delay of one day in the appearance of $PDGF\alpha R^{+}$ cells (Sun et al. 1998). As Pax6 and Nkx2.2 cross-repress one another (Briscoe et al. 2000), it is not surprising that these mice also display a dorsal expansion in the $Nkx2.2^{+}$ domain (Ericson et al. 1997). In fact, this dorsal expansion may explain the OG anomalies seen in the Pax6-deficient mice, since Nkx2.2 represses *Olig2* expression at early stages of spinal cord development (Novitch et al. 2001; Zhou et

al. 2001). Support for this statement comes from the finding that both *PDGFαR* and Olig2 expression are lost in the pMN domain at E13.5 in *Nkx6.1*;*Nkx6.2* double mutant mice, which also exhibit a dorsal expansion in the Nkx2.2^{+} domain (Vallstedt et al. 2005). Furthermore, a ventral expansion in *Olig2* expression, as well as an increased number of *PDGFαR*$^{+}$ cells occurs in the absence of *Nkx2.2* (Qi et al. 2001). This ventral expansion of the *Olig2*$^{+}$ pMN domain also appears to occur in Ngn3 mutant mice, which lack Nkx2.2 expression in the spinal cord at E13.5 (Lee et al. 2003).

Nkx2.2's repression of *Olig2* expression appears to be only a transient event. In particular, although Nkx2.2 and Olig2 are initially expressed in adjacent non-overlapping domains, Nkx2.2 expression subsequently expands dorsally such that it partially overlaps with that of Olig2 during oligodendrogenesis (Agius et al. 2004; Fu et al. 2002; Zhou et al. 2001). Recent research findings implicate Shh in this re-patterning, as well as maintaining the expression of Olig2. In particular, blocking hedgehog signaling in E4 chick spinal cord explants abolishes *Olig2* expression and reduces the size of the Nkx2.2^{+} domain (Agius et al. 2004). Interestingly, evidence also exists supporting a role for Shh signaling in the switch from MN to OG development. In particular, a delay of one day in the onset of *PDGFαR* expression occurs in the absence of *Gli2*, a downstream effector in the Shh signaling pathway (Qi et al. 2003). A similar finding is also seen in mice lacking both *Shh* and *Gli3* (Oh et al. 2005); Gli3 acts as a repressor of ventral fates in the absence of *Shh* (Litingtung and Chiang 2000). In turn, these *Shh*-/-;*Gli3*-/- mice exhibit an increase in the number of newly generated neurons, as well as a delay in the down-regulation of *Ngn2* expression in the pMN domain (Oh et al. 2005). In comparison, over-expressing *Shh* in E1.5 chick neural tubes via electroporation results not only in a dorsal expansion in the Nkx2.2^{+} domain but also a dorsal shift in *Ngn2* expression. This, in turn, is accompanied by an earlier onset of OG development at the expense of MNs (Danesin et al. 2006). However, it is unknown whether the switch from neurogenesis to oligodendrogenesis elicited by Shh is actually due to the dorsal expansion of the Nkx2.2^{+} domain or whether other factors are involved. Current data seems to favors the latter since down-regulation of *Ngn1* and Ngn2 expression in the Olig2^{+} pMN domain precedes the overlap with Nkx2.2 expression (Zhou et al. 2001). In turn, this down-regulation of Ngn1/2 is critical for OG specification to occur, since electroporation of chick embryonic

spinal cords with *Olig2* fails to promote ectopic OG specification unless *Ngn*1 and *Ngn2* expression is repressed (Zhou et al. 2001). Furthermore, a significant increase in the number of *PDGFαR*$^{+}$ cells occurs in the spinal cords of E12.5 *Ngn1*;*Ngn2* double mutant mice (Sugimori et al. 2007).

As there is only a transient delay in OG specification in *Gli2*-/- (Qi et al. 2003) and *Shh*-/-;*Gli3*-/- (Oh et al. 2005) mice, other signaling pathways and/or TFs may compensate for Shh signaling in these mice. Evidence does support a role for Notch signaling in the switch from MN to OG development. In particular, constitutive Notch signaling activity during early stages of zebrafish embryonic development results in excess OPCs being found in the spinal cord at the expense of neurons (Park and Appel 2003). Conversely, reduced Notch signaling activity leads to a large excess of neurons and a loss of OPCs in zebrafish embryonic spinal cords (Park and Appel 2003). Interestingly, a similar phenotype is seen in the spinal cords of chick embryos that undergo *in ovo* electroporation with short interfering RNAs (siRNAs) directed against the *NFIA* (Deneen et al. 2006). Indeed, NFIA is required to maintain the expression of *Hes5* (Deneen et al. 2006), which is a downstream target in the Notch signaling pathway (Wang et al. 1998). How Notch signaling favors OG development is uncertain; however, it may involve its ability to repress the expression of *Ngn1* and *Ngn2* (Zhou et al. 2001).

Evidence also exists supporting a role for Sox9, a class E Sox TF, in the switch from neurogenesis to oligodendrogenesis. Specific ablation of *Sox9* expression in nestin-positive neural stem cells (NSCs) results in a significant transient increase in the number of MNs. This, in turn, is accompanied by a significant reduction in the number of OPCs that partially recovers with continued embryonic development (Stolt et al. 2003). Other class E Sox TFs appear to partially compensate for Sox9 in its absence, since OPC numbers are more severely reduced in the spinal cords of Sox8;Sox9- and Sox9;Sox10-double deficient mice (Stolt et al. 2003; Stolt et al. 2005). Further research will be required, however, to determine whether these SoxE TFs favor OG development via down-regulating Ngn1/2 expression or whether other mechanisms are involved.

Recent research suggests that the SoxD TFs, Sox5 and Sox6, may interfere with SoxE function in the spinal cord via competing for binding sites and/or interaction partners (Stolt et al. 2006). This, in turn, may explain why OPCs arise earlier and are

transiently increased in the spinal cords of Sox5;Sox6 double-deficient mice. However, as the number of MNs is not altered in these mutant mice (Stolt et al. 2006), further research will be needed in order to clarify the exact roles of SoxE and SoxD TFs in OG specification, as well as how they relate to one another.

In addition to the pMN domain, OPCs also appear to arise from the ventral progenitor domains p3, as well as p0-p2 (Sugimori et al. 2007) [Figure 2.1A, Table 2.1]. The OPCs derived from the Nkx2.2^{+} p3 domain appear to arise earlier than the oligodendroglial precursors in the Olig2^{+} pMN domain, and can be distinguished from them until E16.5 in the rat. At this time OPCs also appear to arise from the Pax6^{+} p0-p2 domains, although these cells do not express Pax6. In fact, the down-regulation of Pax6 in these cells appears to be important for the onset of OG development in this region since oligodendroglial cells arise earlier in the p0-p2 domains in *Pax6* mutant mice (Sugimori et al. 2007).

Olig2^{+} OPCs can also arise from a distinct region of the neuroepithelium in the dorsal spinal cord at approximately E15 (Cai et al. 2005; Vallstedt et al. 2005). This region appears to correspond to dp3-5, since a proportion of the Olig2^{+} cells are closely associated with Pax7- and Mash1-neuroepthelial expression, which overlaps in these progenitor domains. Furthermore, Olig2^{+} cells are found immediately dorsal to the *Dbx2*$^{+}$ domain, which delineates the dp5/dp6 boundary (Cai et al. 2005) [Figure 2.1A]. However, as new research suggests that OG specification in the dorsal spinal cord may be more extensive than this (Battiste et al. 2007), further investigations will be needed.

In contrast to its ventral counterpart, OG specification in the dorsal spinal cord seems to occur independently of Shh signaling since *Olig2*$^{+}$ cells are found in this region in E14.5 *Shh* mutant mice (Cai et al. 2005). Instead, OG development in this region is believed to occur when BMP signaling is sufficiently reduced with continued embryonic development. Accordingly, treatment of dorsal spinal cord explants with BMP7 inhibits Olig2 expression (Vallstedt et al. 2005), which is critical for OG development throughout the spinal cord (Lu et al. 2002).

2.1.1.2 Hindbrain

In the ventral hindbrain, OPCs arise in one of two specific progenitor domains depending upon the anterior-posterior location. In the most posterior hindbrain, OPCs

arise in the Olig2$^+$ pMN domain similar to that seen in the ventral spinal cord (Vallstedt et al. 2005). In contrast, in the anterior hindbrain, which lacks a pMN domain (Pattyn et al. 2003), OPCs arise in the Nkx2.2$^+$ pMNv domain located just dorsal to the FP (Vallstedt et al. 2005) [Figure 2.1B].

The regulation of OG specification in the ventral hindbrain differs from that of the spinal cord in several respects, which may or may not be domain specific. For instance, unlike the spinal cord, Olig2 is not critical for OG specification in the murine hindbrain since early stages of OG development occur largely unaltered in its absence (Lu et al. 2002). Instead, Olig1 appears to compensate for Olig2 in this region, since PDGFαR expression is absent in the hindbrains of *Olig1*;*Olig2* double knockout mice (Zhou and Anderson 2002). Although the OG anomalies seen in these mutant mice do not appear to be domain specific, further research will be needed to verify this.

In comparison, evidence does suggest that Nkx2.2 differentially affects OG specification depending upon the anterior-posterior region of the hindbrain and, hence, the specific ventral progenitor domain. Similar to the spinal cord, a dorsal expansion of the Nkx2.2$^+$ domain is accompanied by a loss of PDGFαR$^+$ cells in the posterior hindbrain of *Nkx6.1*;*Nkx6.2* mutant mice (Pattyn et al. 2003; Vallstedt et al. 2005). In contrast, in the anterior hindbrain of these mutant mice, this dorsal expansion corresponds with extensive ectopic PDGFαR expression (Vallstedt et al. 2005). Hence, unlike the spinal cord and posterior hindbrain, Nkx2.2 appears to promote OG specification in the anterior hindbrain, where OPCs arise from the unique pMNv domain.

With the exception of rhombomere (r) 1, dorsal *Olig1*$^+$ OPCs arise at E13.5 in a region of the hindbrain neuroepithelium immediately dorsal to the *Dbx2*$^+$ domain. Although very little is known regarding the regulation of OG specification in the dorsal hindbrain, it appears to be similar to that of the dorsal spinal cord (Vallstedt et al. 2005).

2.1.1.3 Telencephalon

In the ventral telencephalon, OPCs arise in two distinct waves: first, from the MGE, as well as the anterior entopenduncular area (AEP) and, then, subsequently from the LGE and/or caudal ganglionic eminence (CGE) (Kessaris et al. 2006) [Figure 2.1C]. Like the ventral spinal cord, OG specification in this region is Shh-dependent, since blocking hedgehog signaling strongly inhibits OG development in ventral forebrain

cultures (Tekki-Kessaris et al. 2001). Furthermore, *PDGFαR* expression is absent in the forebrain of 13.5 days post coitum (dpc) *Shh* mutant mice (Alberta et al. 2001).

Despite this similarity, however, several differences have been identified regarding the regulation of OG specification in this region. For example, unlike the spinal cord, Olig2 does not appear to be critical for OG specification in a focal region of the ventral forebrain, since *PDGFαR* expression is evident in its absence (Lu et al. 2002). Instead, Olig1 appears to compensate for Olig2 in this region, as evident by the fact that all examined brain regions lack *PDGFαR*$^+$ cells in *Olig1*;*Olig2* double knockout mice (Zhou and Anderson 2002).

The neuroepithelium of the ventral telencephalon, unlike the pMN domain, expresses a number of distinct TFs, including Nkx2.1, Gsh2 (Corbin et al. 2003; Kessaris et al. 2006), and Mash1 (Corbin et al. 2003; Porteus et al. 1994) [Figure 2.1C]. Interestingly, evidence suggests that each of these TFs affects OG specification in this region. In particular, Nkx2.1 appears to promote OG specification in the ventral telencephalon since a transient delay in the appearance of *PDGFαR* expression occurs in its absence (Nery et al. 2001; Tekki-Kessaris et al. 2001). Whether its effect is direct or indirect is unknown; however, current evidence favors the latter since *Shh* expression is largely absent in the ventral telencephalon of *Nkx2.1* mutant mice (Sussel et al. 1999; Tekki-Kessaris et al. 2001). Recent research also suggests that Mash1 is important for OG specification in the MGE/AEP since in its absence a dramatic transient decrease in PDGFαR expression is observed in the ventral telencephalon at E12.5 and E13.5 (Parras et al. 2007). In contrast, Gsh2 appears to inhibit OG specification, at least in certain ventral telencephalic regions, since in its absence ectopic *PDGFαR* expression is found in the neuroepithelium of the MGE, as well as in the ventral-most LGE (Corbin et al. 2003). Although the reason for this ectopic OG development is unknown, it may be associated with patterning defects seen in the ventral telencephalon of these mutant mice (Corbin et al. 2003).

In the dorsal telencephalon, OPCs arise from the neuroepithelium, which is characterized by the expression of the TF Emx1 (Kessaris et al. 2006). OG specification in this area, like several other CNS regions, appears to be inhibited by BMP signaling since treatment of E16 rat cortical cultures with BMP2 significantly decreases the number

of oligodendroglial cells (Mabie et al. 1999). In comparison, Shh signaling appears to promote OG development in this region since blocking hedgehog signaling in E15.5 cortical explants strongly inhibits oligodendrogenesis (Tekki-Kessaris et al. 2001). In contrast, Olig2 does not appear to be critical for OG specification in the dorsal telencephalon since *PDGFαR*$^{+}$ cells are found when *Olig2* expression is specifically ablated in cortical progenitors (Yue et al. 2006). Although it is currently unknown whether Olig1 could compensate for Olig2 in this region, it appears to be a distinct possibility since the percentage of OPCs is greatly increased when cortical cultures are infected with an *Olig1*-containing adenovirus (Alberta et al. 2001; Lu et al. 2000).

2.1.1.4 Conclusion

Over the past decade enormous strides have been made in our understanding of the regulation of oligodendrogenesis, especially in the ventral spinal cord. In this region OG specification is largely the result of the interplay between Shh, BMP, and Notch signaling pathways. In turn, these pathways regulate the expression of the TFs that dictate the spatial and/or temporal aspects of oligodendrogenesis [Figure 2.3]. Although much less is known regarding the regulation of OG specification in other ventral and/or dorsal regions of the spinal cord, hindbrain, and telencephalon, several similarities, as well as differences have been identified. Interestingly, the differences appear to be partially related to the TFs involved in patterning these distinct progenitor domains. Hence, identifying other TFs and signaling pathways in these neuroepithelial domains may greatly expedite research into the regulation of OG specification in these CNS regions. Ultimately, understanding similarities, as well as differences regarding the transcriptional control of oligodendrogenesis in specific CNS regions will aid in the development of therapeutic treatments for demyelinating diseases, such as multiple sclerosis (MS).

2.1.2 Differentiation

Following specification, OG development *in vitro* progresses through four distinct stages characterized by unique antigenic and morphological phenotypes [Figure 2.4]. In particular, during the first stage of oligodendrogenesis, known as the OPC stage, the cells are bipolar and express a complex ganglioside on their surface that is recognized by the monoclonal antibody A2B5 (Abney et al. 1983; Eisenbarth et al. 1979; Wang et al. 1998).

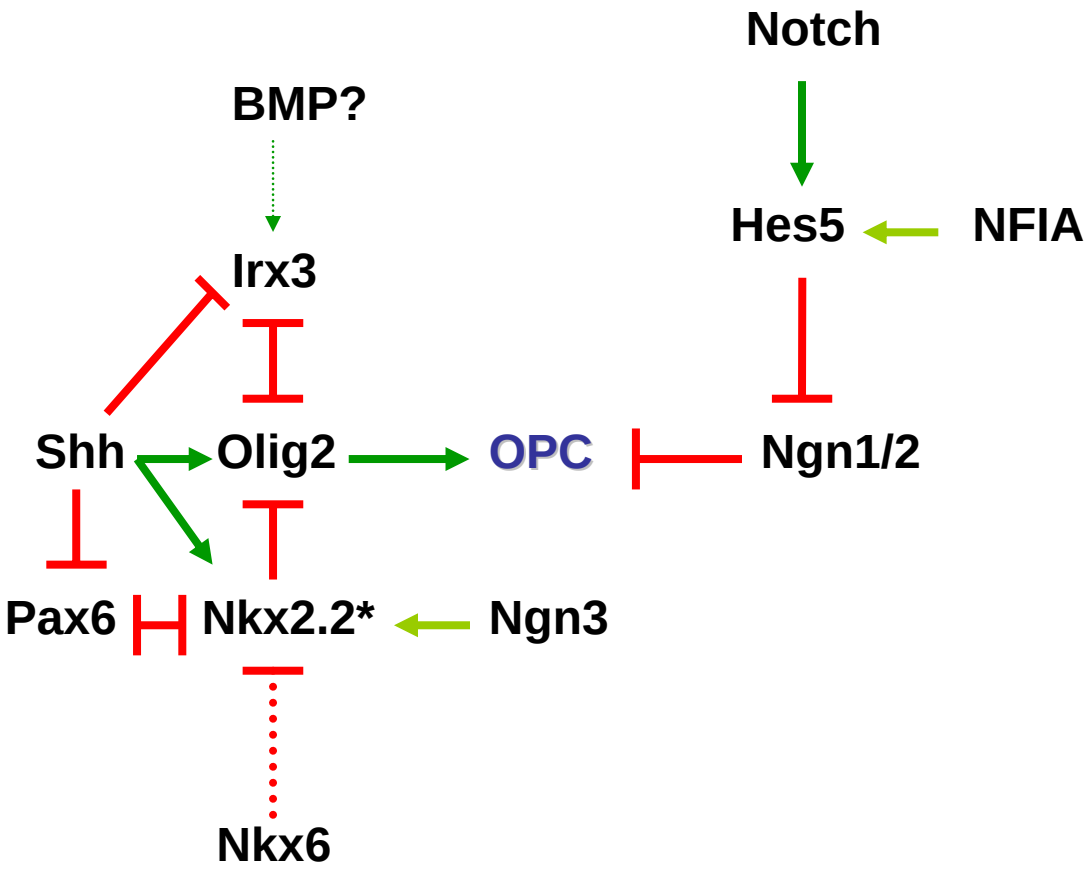


Figure 2.3 Basic transcriptional cascade of oligodendrocyte specification in the pMN domain.

Many TFs (eg.Olig2, Nkx2.2, Pax6) and signaling pathways (BMP, Notch, Shh) are important for OG specification in the pMN domain. Interestingly, many of these factors are involved in the dorsal-ventral patterning of the spinal cord. For instance, Shh directs the formation of five ventral progenitor domains, including the pMN domain, via regulating the expression of TFs in the vVZ. This includes regulating the expression of Nkx2.2 and Pax6, as well as *Olig2* and *Irx3*, which define the ventral and dorsal boundaries, respectively, of the pMN domain. Subsequently, cross-repressive interactions between these TFs maintain and refine these boundaries. Of these TFs only Olig2 is critical for OG specification in this region; however, it cannot promote OG development unless *Ngn1/2* expression is down-regulated such as via Notch signaling.

Abbreviations: ?- speculated; *-transient effect; →- enhance; ⊣-repress; →-maintain

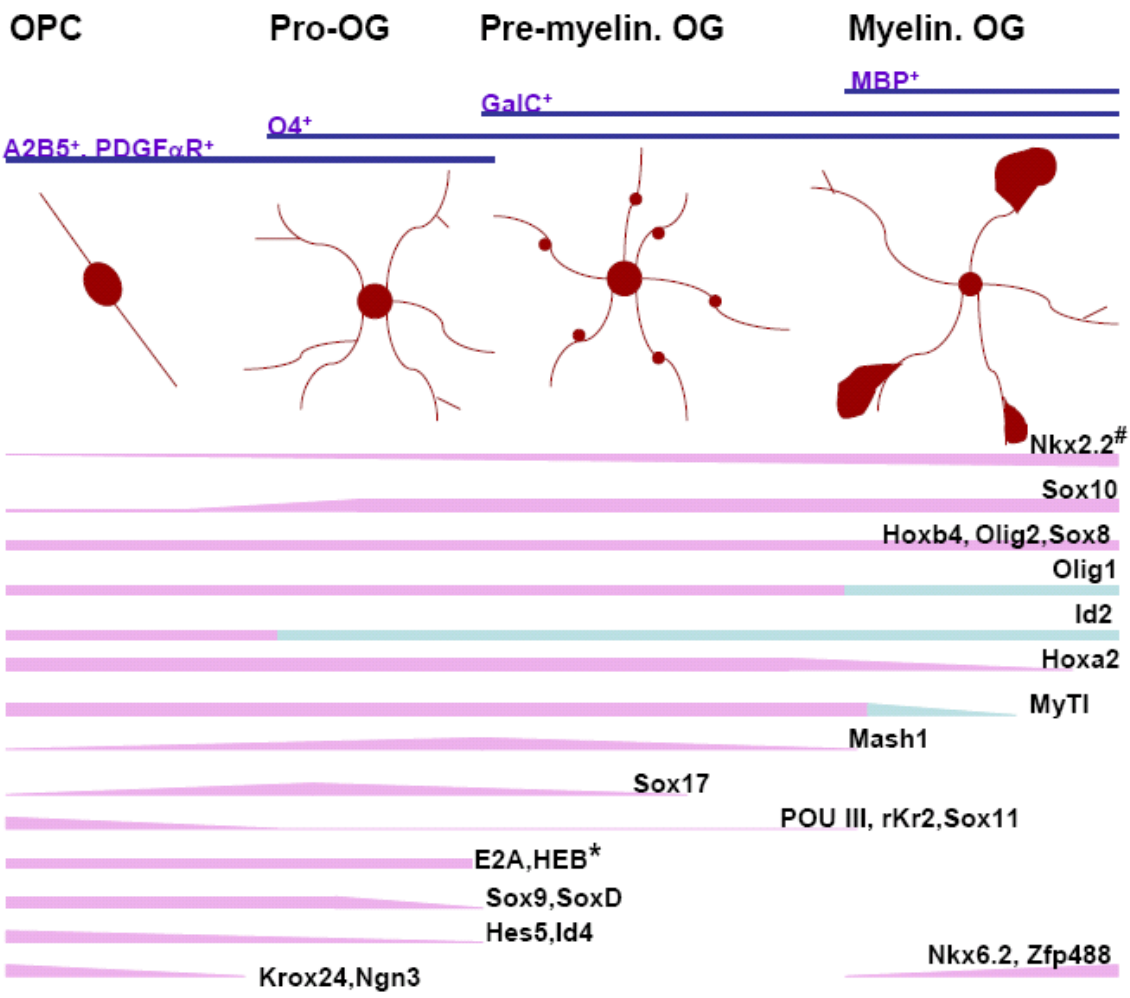


Figure 2.4 Expression profiles of various transcription factors during oligodendrocyte development.

(top) OGs progress through four distinct stages characterized by unique morphological and antigenic phenotypes. (myelin.: myelinating)

(bottom) Speculated expression profiles of the TFs expressed by cells of the oligodendroglial lineage. Profiles for individual TFs were determined via an analysis of published data that utilized immunocytochemical, immunohistochemical, RT-PCR, *in situ* hybridization, and Northern blot analyses. (See Table 2.2 for references). If not stated otherwise, expression was assumed to be nuclear. In cases where the overall expression profiles of two or more TFs appeared to be similar, these TFs were grouped together (ex. Hes5 and Id4). Variations in line thickness illustrate either changes in the percentage of cells expressing the TF(s) or the intensity of the TF(s) expression.

(▬) nuclear expression; (▬) cytoplasmic expression; POU III: Brn-1,2 and SCIP;*later stages of OG development were not analyzed; # expression profile may vary depending on CNS region or cell line.

Progression of these cells to the pro-OG stage occurs when they become multipolar and express surface epitopes recognized by the O4 monoclonal antibody (Bansal et al. 1989; Gard and Pfeiffer 1989). Subsequently, terminal differentiation into pre-myelinating OGs is signified by the emergence of GalC expression (Fok-Seang and Miller 1994; Noble et al. 1988). Progression to the mature myelinating phenotype occurs when the cells begin to synthesize myelin proteins, such as myelin basic protein (MBP) and proteolipid protein (PLP), and elaborate sheet-like membranes (Duchala et al. 1995; Gard and Pfeiffer 1989).

In the developing embryo, OPCs initially arise in multiple distinct foci located in various regions of the CNS (Cai et al. 2005; Perez Villegas et al. 1999; Pringle and Richardson 1993; Spassky et al. 1998). Subsequently, these cells must proliferate and migrate out of these foci in order to populate specific CNS regions (Ono et al. 1997; Pringle and Richardson 1993; Spassky et al. 1998; Tekki-Kessaris et al. 2001). As pre-myelinating OGs do not actively proliferate (Fok-Seang and Miller 1994) or migrate (Noble et al. 1988), the proper progression of oligodendroglial cells through the various stages of development is critical to ensure normal myelination and, hence, CNS functioning.

As the oligodendroglial cells progress from precursor to mature myelinating cells they express a dynamic combination of TFs [Figure 2.4]. Whereas some TFs, such as Sox10 (Stolt et al. 2002), are expressed throughout OG development, the expression of many TFs, such as SCIP (Collarini et al. 1992) and Krox24 (Sock et al. 1997), is transient. In most cases their expression decreases with differentiation (Collarini et al. 1992; Schreiber et al. 1997); however, *Nkx6.2* and *Zfp488* expression increase in time with that of myelin protein genes (Awatramani et al. 1997; Wang et al. 2006).

Over the last decade the role(s) of some of these TFs has been elucidated utilizing transgenic knockout mice, as well as transfection assays [Table 2.2]. Most of these TFs are known downstream targets/effectors of the Shh, BMP or Notch signaling pathways. Therefore, the purpose of this section of the literature review is to describe signaling pathways and TFs that inhibit and/or promote this process. As OG differentiation appears to be regulated similarly in the brain and spinal cord (Genoud et al. 2002; Xin et al. 2005), its regulation in these regions will be examined in unison.

Table 2.2 Transcription factors expressed by cells of the oligodendrocyte lineage.

Family	Class/ Group	Transcription Factor	Role in OG Differentiation	Additional CNS Expressing Cells
HLH	I	E2A[36]		
		HEB[36]		
	II	Mash1[12,39]		Neural precursor[18]
		Ngn3[17]	+[17]	Neural precursor[17]
		Olig1[4,21]	+[20,40]	Neural precursor[21]
		Olig2[4,8]	+[8]	Neural precursor[21,26]
	V	Id2[39]	-[13,39]	Neural precursor, neuron[11,22], astrocyte[37]
		Id4[13]		Neural precursor, neuron[11]
	VI	Hes5[12]	-[12]	Neural precursor, neuron[1]
Hox	A	Hoxa2[9,23]	-^[41]	Neuron[9,23], astrocyte[9]
	B	Hoxb4[24]		Neuron[24]
Nkx		Nkx2.2*[27]	+[27]	Neural precursor[27]
		Nkx6.2*[5]	Myelin (u)[31]	Neural precursor, neuron[31]
POU	III	Brn-1[28]		Neural precursor[2], neuron[10]
		Brn-2[28]		Neural precursor[2], neuron[10]
		SCIP[7,28]		Neural precursor, neuron[2]
Sox	C	Sox4[15]		Neural precursor, neuron[6]
		Sox11[15]		Neural precursor, neuron[6,15]
	D	Sox5[32]	-[32]	Neural precursor , neuron, radial glia[32]
		Sox6[32]	-[32]	Neural precursor , neuron, radial glia[32]
	E	Sox8*[33]	+[33]	
		Sox9[34]		Neural precursor, radial glia, astrocyte[34], Bergmann glia[14]
		Sox10[16]*[35]	+[35]	
	F	Sox17[30]	+[30]	Neuron, astrocyte[30]
Zinc Finger		Krox24[29]		
		MyTI[3]*[25]	+[25]	Neural precursor, neuron[3]
		rKr2[19]		Neural precursor,neuron[19]
		Zfp488[38]	+[38]	

Abbreviations: HLH: helix-loop-helix; +: promote; -: inhibit; *: TF binding sites found in the promoters of myelin protein genes; (u): ultrastructure; ^: data obtained from CG4 cells

References: [1]Akazawa et al. (1992) , [2]Alvarez-Bolado et al. (1995), [3]Armstrong et al. (1995), [4]Arnett et al. (2004), [5]Awatramani et al. (1997), [6]Cheung et al. (2000), [7]Collarini et al. (1992), [8] Fu et al. (2002), [9]Hao et al. (1999), [10]He et al. (1989), [11]Jen et al. (1996), [12]Kondo and Raff (2000a), [13]Kondo and Raff (2000b), [14]Kordes et al. (2005),[15]Kuhlbrodt et al. (1998a), [16]Kuhlbrodt et al. (1998b), [17]Lee et al. (2003), [18]Lo et al. (1991), [19]Lovas et al. (2001), [20]Lu et al. (2002), [21] Lu et al. (2000), [22]Neuman et al. (1993) [23]Nicolay et al. (2004a), [24] Nicolay et al. (2004b), [25]Nielsen et al. (2004), [26]Novitch et al. (2001), [27]Qi et al. (2001), [28]Schreiber et al. (1997), [29]Sock et al. (1997), [30]Sohn et al. (2006), [31]Southwood et al. (2004), [32]Stolt et al. (2006), [33]Stolt et al. (2004), [34]Stolt et al. (2003), [35]Stolt et al. (2002), [36]Sussman et al. (2002), [37]Tzeng and De Vellis (1998), [38]Wang et al. (2006), [39]Wang et al. (2001), [40]Xin et al. (2005), [41] Section 6.3.3, pg. 90

2.1.2.1 Inhibitory factors

Following specification, OPCs must migrate out from their neuroepithelial domains in order to populate specific CNS regions. As a result, their differentiation must be inhibited until they reach their final destination. A number of signaling pathways have been implicated in this inhibition including Notch and BMP. Most of the evidence supporting the role of Notch signaling in this process has resulted from manipulating one specific Notch receptor, Notch1. In particular, activation of the Notch signaling pathway in purified OPC cultures, either by exposure to standard Notch ligands or by transfection with a vector encoding a constitutively active form of *Notch1*, results in a large proportion of these cells failing to differentiate (Wang et al. 1998). Similarly, induction of a constitutively active form of zebrafish *Notch1a* following OG specification greatly reduces the number of spinal cord oligodendroglial cells that progress to the *plp1*/*dm20*$^{+}$ stage (Park and Appel 2003). In contrast, the expression of PLP and MBP is transiently increased in numerous brain regions of *Notch1*-deficient mice (Givogri et al. 2002). Furthermore, specific ablation of *Notch1* in oligodendroglial cells results in premature differentiation in the murine spinal cord and cerebrum (Genoud et al. 2002). The mechanism by which Notch signaling inhibits OG differentiation has not been determined; however, it may involve its ability to enhance the expression of *Hes5* (Wang et al. 1998). Indeed, over-expression of *Hes5* in purified OPCs results in most cells failing to differentiate (Kondo and Raff 2000a).

Hes5 appears to inhibit OG differentiation via multiple mechanisms. In particular, it can directly repress the expression of Sox10, which is important in this process. In addition, it can repress MBP promoter activity in Oli-neu cells (Liu et al. 2006); this effect is most likely indirect since Hes5 binding to the MBP promoter is not required for its repression of promoter activity. The mechanism by which Hes5 represses MBP expression is unknown but may involve its sequestration of Sox10 and/or Mash1 (Liu et al. 2006), two TFs known to activate the MBP promoter in Oli-neu cells (Gokhan et al. 2005). Binding of Hes5 to Sox10 and/or Mash1 may interfere with their ability to enhance MBP expression. This, in turn, may explain why Hes5 is only able to repress MBP promoter activity when *Sox10* is expressed at low levels (Liu et al. 2006). Sox10 expression levels appear to increase after primary oligodendroglial cells are induced to

differentiate *in vitro* (Dugas et al. 2006; Wei et al. 2004). As a result, Hes5's ability to interfere with Sox10's function should be limited to early stages of oligodendrogenesis.

Evidence also suggests that BMP signaling inhibits OG differentiation. Placement of BMP4-soaked beads at the ventral midline of murine cervical spinal cord/hindbrain explants results in a dramatic decrease in PLP^+ and myelin associated glycoprotein $(MAG)^+$ cells in the area around the beads (See et al. 2004). The mechanism by which BMP signaling accomplishes this inhibitory effect is unknown, but may involve its ability to induce the expression of the TFs Id2 and Id4 (Samanta and Kessler 2004). Accordingly, over-expression of *Id2* (Wang et al. 2001) or *Id4* (Kondo and Raff 2000b) in purified OPCs results in most cells failing to differentiate into $GalC^+$ OGs. Although the reason for their inhibitory effect is unknown, it may be due to their proposed ability to prevent heterodimerization of Olig TFs with E proteins (Samanta and Kessler 2004), which, in turn, are believed to mediate Olig TF effects (Sussman et al. 2002), such as promoting OG differentiation (Lu et al. 2002; Xin et al. 2005).

The Sox D TFs, Sox5 and Sox6, are also important for inhibiting OG differentiation since a significant increase in the number of *MBP*- and *PLP*-expressing cells is evident in the spinal cords of 18.5 dpc Sox5;Sox6 conditional-double deficient mice (Stolt et al. 2006). It appears that these TFs inhibit OG differentiation via interfering with the function of Sox10. In particular, Sox5 and Sox6 can block Sox10-directed activation of the MBP promoter in oligodendroglial cell lines. As these TFs can recognize Sox10-binding sites and interaction partners (Stolt et al. 2006), they may block Sox10 function via multiple mechanisms.

2.1.2.2 Promoting factors

Various TFs, including Olig1, Olig2, Nkx2.2 and MyTI, favor OG differentiation. For example, in *Olig1* mutant mice, *PLP/DM20* and *MBP* expression is markedly reduced in the postnatal day (P) 0 (Lu et al. 2002) and P14 (Xin et al. 2005) spinal cord and undetectable in the P14 corpus callosum, neocortex and striatum (Xin et al. 2005). Similarly, the number of $GalC^+$ and PLP^+ OGs is significantly reduced in spinal cord cultures following *Olig2*-antisense treatment (Fu et al. 2002). Olig1 and Olig2 may promote OG differentiation by regulating the expression of other TFs. One such TF is Zfp488, which is a potential downstream target of Olig1. *Olig1* mutant mice lack *Zfp488*

expression in the spinal cord, optic nerves, cerebellum, and corpus callosum. In turn, down-regulating *Zfp488* expression in the oligodendroglial cell line CG4 results in a reduction in *MBP* and 2′,3′-cyclic nucleotide 3′-phosphodiesterase (*CNP*) gene expression (Wang et al. 2006). Interestingly, *Zfp488*'s transcription appears to be specifically regulated by Olig1 since only Olig1, not Olig2, can significantly enhance luciferase reporter activity driven by its putative promoter. Olig2 does appear to interact with Zfp488 at the protein level (Wang et al. 2006); however, further research will be needed to determine if this interaction is required for their function.

Although Olig2 does not appear to regulate Zfp488 expression, evidence suggests that it can affect *Sox10* expression. Ectopically expressing *Olig2* in chick embryonic spinal cords via *in ovo* electroporation induces *Sox10* expression (Liu et al. 2007). In contrast, *Sox10* mRNA is undetectable throughout the spinal cord in the absence of *Olig2* (Lu et al. 2002). In comparison, *Olig1*, whose expression reappears after *Sox10* in the rodent embryonic spinal cord (Zhou et al. 2000), may be important in the maintenance of *Sox10* expression since *Olig1* mutant mice exhibit a decrease in the number of $Sox10^+$ cells (Lu et al. 2002; Xin et al. 2005). As researchers have yet to find a chick *Olig1* ortholog (Zhou et al. 2001), this may explain why strong expression of *Sox10* is seen much earlier in rodents (Zhou et al. 2000) than in the chick (Zhou et al. 2001).

Sox10 is important in OG differentiation since there is a delay and a dramatic decrease in *PLP* and *MBP* expression in the spinal cords of Sox10-deficient mice (Stolt et al. 2002). Furthermore, transplantation of Sox10-deficient NSCs into 3 to 9 day old mouse retinas fails to myelinate the host nerve fiber layer (Stolt et al. 2002). More recently, Dugas et al. (2006) used siRNA to knockdown *Sox10* expression in OPCs *in vitro* and found a significant reduction in the number of cells that differentiate into MBP-expressing OGs.

In comparison, over-expressing *Sox10* in embryonic chick spinal cords via *in ovo* electroporation induces the expression of Nkx2.2, Olig2, *MBP*, *PLP* and MAG (Liu et al. 2007). As some of the MAG^+ cells co-express Nkx2.2 or Olig2 (Liu et al. 2007), it suggests that Sox10 may indirectly enhance myelin protein gene expression through these TFs. However, as a proportion of the MAG^+ cells do not express Nkx2.2 or Olig2 (Liu et al. 2007), Sox10 may also induce myelin protein gene expression directly. Indeed, Sox10

does appear to directly regulate MBP expression since it can bind to three distinct sites in MBP's proximal promoter. In turn, Sox10-directed activation of this proximal promoter is reduced or almost completely blocked by mutating the three sites individually or all together, respectively (Stolt et al. 2002).

Sox10 also appears to regulate the transcription of two gap junction proteins, connexin47 (Schlierf et al. 2006) and connexin32 (Bondurand et al. 2001; Schlierf et al. 2006), which are important for normal CNS myelination (Menichella et al. 2003). In particular, Sox10 activates the connexin32 promoter (Bondurand et al. 2001; Schlierf et al. 2006), as well as the connexin47 promoter 1b (Schlierf et al. 2006) in luciferase reporter gene assays. This activation appears to be direct since it is dramatically reduced by mutations in either the Sox10 DNA-binding domain or binding sites (Bondurand et al. 2001; Schlierf et al. 2006). In turn, these connexin proteins are important for normal CNS myelination since *connexin32*;*connexin47* double knockout mice exhibit profound CNS myelin abnormalities, such as thin, vacuolated, or absent myelin sheaths (Menichella et al. 2003).

Since Sox10 is a poor transcriptional effector on its own (Kuhlbrodt et al. 1998b), other TFs are likely implicated in this process. One potential candidate is the TF SCIP which is expressed by oligodendroglial cells (Collarini et al. 1992) and functions synergistically with Sox10 (Kuhlbrodt et al. 1998b). However, myelination occurs normally in the spinal cords of *SCIP* mutant mice (Bermingham Jr. et al. 1996). In contrast, mice lacking the ligand-dependent TF *COUP-TFI*, which is a potential upstream regulator of *SCIP*, exhibit CNS hypomyelination and dysmyelination (Yamaguchi et al. 2004). The reason for the disparity in these mutant phenotypes is unknown but may reflect the fact that two additional class III POU TFs, Brn-1 and Brn-2, which could be regulated by COUP-TFI, are expressed by oligodendroglial cells (Schreiber et al. 1997) and, hence, could compensate for SCIP in its absence. Interestingly, oligodendroglial cells express two additional Sox genes, *Sox4* and *Sox11*, which function synergistically with Brn-1 and/or Brn-2 (Kuhlbrodt et al. 1998a).

Two additional TFs that appear to function synergistically with Sox10 are Olig2 and Sp1. When *Olig2* and its heterodimerization partner *E47* are co-transfected with *Sox10* in Oli-neu cells there is a synergistic increase in MBP promoter activity (Gokhan

et al. 2005). As Sox10 can interact with Olig2 at the protein level (Stolt et al. 2006), this synergistic activation may depend on the formation of a transcriptional complex. Like Olig2, a synergistic activation of the MBP promoter is also seen when NIH 3T3 cells are co-transfected with *Sox10* and *Sp1* (Wei et al. 2004); however, it is unknown whether these TFs interact at the protein level.

Sox8, another group E Sox TF, also plays a role in OG differentiation since a transient reduction occurs in the number of MBP^+ and PLP^+ cells in the spinal cords of Sox8-deficient mice (Stolt et al. 2004). Hence, the myelin protein expression seen in Sox10-deficient mice may be due to the compensatory effects of Sox8 and vice versa. In fact, Sox8 appears to have a limited ability to compensate for Sox10 since a dramatic decrease in *MBP* and *PLP* expression is found in the spinal cords of P7 transgenic mice in which Sox10 is replaced by Sox8 (Kellerer et al. 2006).

In addition to Sox10, Olig2 also appears to regulate the expression of Nkx2.2. In particular, ectopic Nkx2.2 expression is found five days following *in ovo* electroporation of E2 chick spinal cords with an *Olig2*-expressing vector (Liu et al. 2007). Nkx2.2 is important in promoting OG differentiation since a delay and a dramatic decrease in *PLP/DM20* and *MBP* expression is evident in the spinal cords of *Nkx2.2* mutant mice (Qi et al. 2001). Furthermore, in its absence, *MBP* expression is reduced or lost in the E17.5 medulla and P7 forebrain, respectively (Qi et al. 2001). Correspondingly, *Ngn3* mutant mice, which lack Nkx2.2 expression in the E13.5 spinal cord, display a dramatic reduction in *PLP* and *MBP* expression at P0 (Lee et al. 2003).

How Nkx2.2 promotes OG differentiation is not clear, but it appears to involve its ability to regulate the expression of certain myelin protein genes. In particular, over-expressing *Nkx2.2* in NIH 3T3 cells can induce green fluorescent protein expression driven by a portion of the PLP promoter (Qi et al. 2001). This induction may be direct since consensus Nkx2.2-binding sites are found in the PLP promoter (Qi et al. 2001). The MBP promoter also contains Nkx2.2-binding sites (Qi et al. 2001). In contrast to PLP, however, Nkx2.2 represses MBP promoter activity either on its own (Wei et al. 2005) or that directed by certain other TFs, including Sox10 (Gokhan et al. 2005). Although these results appear to contradict the transgenic knockout data, they may reflect a cell line- or CNS region-specific effect. In particular, these transient transfection assays

were conducted utilizing two oligodendroglial cell lines, CG4 (Wei et al. 2005) and Oli-neu (Gokhan et al. 2005). The CG4 and Oli-neu cell lines were established from primary oligodendroglial cultures obtained from either the perinatal rat cerebral cortex (Louis et al. 1992) or E15 murine brains (Jung et al. 1995), respectively. Interestingly, Nkx2.2's expression profile during OG development appears to vary not only between these two cell lines, but also with that of primary cultures obtained from the embryonic murine spinal cord (Fu et al. 2002; Gokhan et al. 2005; Qi et al. 2001; Wei et al. 2005). Resolution of this issue will have to await further research.

Another Nkx gene, *Nkx6.2*, exhibits an expression profile similar to that of *MBP* and *PLP* (Awatramani et al. 1997). Although the MBP and PLP promoters contain multiple Nkx6.2-binding sites (Awatramani et al. 1997), *MBP* is similarly expressed in the spinal cord, forebrain, and cerebellum of both wild-type (WT) and *Nkx6.2* mutant mice (Cai et al. 2001). However, Nkx6.2 is required for normal myelin ultrastructure (Southwood et al. 2004).

Evidence suggests that the TF MyTI may also promote OG differentiation. Oligodendroglial cells co-transfected with a plasmid expressing the seven zinc-finger isoform of MyTI and a CNP-luciferase reporter results in a 6.7-fold induction in reporter activity compared to the empty vector (Nielsen et al. 2004). Furthermore, infection of rat OPC cultures with a retrovirus containing a truncated MyTI, which is designed to interfere with endogenous MyTI function, results in a significant reduction in the differentiation of oligodendroglial cells (Nielsen et al. 2004).

Sox17, a Sox F TF, is also important in OG differentiation (Sohn et al. 2006). Down-regulation of *Sox17* expression in purified OPCs with siRNAs results in a significant increase or decrease in the percentage of $A2B5^+$ and $O4^+$ cells, respectively. Not only are these findings reversed in OPCs which over-express *Sox17*, but these cells also display increased RNA levels of *MBP*, *MAG*, and *CNP*. In turn, Sox17 may promote OG differentiation via several mechanisms. For instance, over-expression of *Sox17* in purified OPCs results in a significant decrease in the percentage of proliferative cells, which suggests that it could promote cell cycle exit. In addition, Sox17 may regulate the transcription of particular myelin protein genes. It can enhance luciferase activity driven

by the MBP promoter (Sohn et al. 2006); however, whether this effect is direct or indirect will require further research.

Surprisingly, recent research suggests that Notch signaling also promotes OG differentiation (Cui et al. 2004; Hu et al. 2003). Although unexpected given the previous information [Section 2.1.2.1, pg.18], it could reflect a ligand-, receptor-, and/or downstream effector-dependent event. Indeed, evidence suggests that it could be ligand-dependent. Whereas activation of Notch signaling via the standard Notch ligands, Jagged1 or Delta1, results in most OPCs failing to differentiate (Hu et al. 2003; Wang et al. 1998), treatment of purified OPCs with the novel Notch ligands, F3 or NB-3, promotes their maturation into CNP^+ OGs (Cui et al. 2004; Hu et al. 2003). In contrast, current data does not support a receptor-dependent effect since Notch1 appears to be important for inhibiting (Genoud et al. 2002; Givogri et al. 2002), as well as promoting (Cui et al. 2004; Hu et al. 2003) OG differentiation. However, as *Notch2* and/or *Notch3* are expressed in similar areas as *Notch1* in the embryonic and postnatal CNS (Irvin et al. 2001; Lindsell et al. 1996), further research will be needed to clarify which receptors are involved in each process. In comparison, there is evidence to support a downstream effector-dependent event. In particular, the Delta1/Notch signaling pathway in OPCs appears to utilize CBF1 (Wang et al. 1998), whereas the F3,NB-3/Notch signaling pathway in the same cells requires Deltex1 (Cui et al. 2004; Hu et al. 2003). Interestingly, these Notch signaling pathways also appear to activate distinct target genes since exposing oligodendroglial cells to Delta1 or F3/NB-3 enhances the mRNA expression of *Hes5* (Wang et al. 1998) or *MAG* (Cui et al. 2004; Hu et al. 2003), respectively. Hence, various factors may dictate what effect Notch signaling has on OG differentiation.

2.1.2.3 Conclusion

Over the past decade researchers have made significant contributions to our understanding of the transcriptional control of OG differentiation [Table 2.2]. In most cases, the TFs and signaling pathways identified either inhibit or promote this process; however, instances exist where the effect of a particular signaling pathway may depend upon such factors as the ligand and/or downstream effector. Intriguingly, many of the signaling pathways and TFs appear to act, in part, via affecting the expression and/or

function of other TFs [Figure 2.5]. In addition, many appear to play similar roles throughout the CNS. However, it should be kept in mind that some of this information was obtained utilizing *in vitro* assays (Barres et al. 1994; Cui et al. 2004; Hu et al. 2003; Laeng et al. 1994; Nielsen et al. 2004; Noll and Miller 1994; See et al. 2004; Sohn et al. 2006) and, hence, will have to be verified *in vivo*. In addition, further research will be required to clarify where particular TFs play a role in OG differentiation since most researchers have only examined the expression of myelin proteins. Furthermore, it remains to be determined how the transcriptional control of OG differentiation compares between ventral and dorsal progenitors. Although this is a huge undertaking, it will be aided by the vast knowledge already accumulated regarding the expression of TFs in the CNS [Table 2.3]. The importance of obtaining this information is evident from the findings that although ventral and dorsal OPCs appear to be able to compensate for each other if one progenitor type is ablated (Cai et al. 2005; Kessaris et al. 2006), this compensation appears to be limited if the OPCs are present but their maturation is defective (Yue et al. 2006). Ultimately, identifying the molecular mechanisms regulating OG development will be critical to the establishment of therapeutic strategies for demyelinating diseases, such as MS.

2.2 Introduction to *Hox* genes in the central nervous system: *Hoxa2*

Hox genes, which exhibit sequence homology to *Drosophila* homeotic complex genes, can be identified by the presence of a 180 base pair homeobox that encodes a 60 amino acid homeodomain (McGinnis et al. 1984a; McGinnis et al. 1984b). Through their homeodomain Hox TFs can bind to specific nucleotide sequences and affect the expression of downstream effector genes (Akin and Nazarali 2005; Hirsch et al. 1990; Hoey and Levine 1988; Jones et al. 1992). In turn, the binding specificity of Hox TFs *in vivo* often depends upon the presence of co-factors, such as Pbx and Prep/Meis (Moens and Selleri 2006).

In mice and humans, 39 *Hox/HOX* genes have been identified that can be arranged into four clusters (Hox A, B, C, D) found on different chromosomes (Akin and Nazarali 2005; Favier and Dollé 1997; Santagati and Rijli 2003; Scott 1992) [Figure 2.6]. The location of a particular *Hox* gene within a cluster determines its anterior limit of

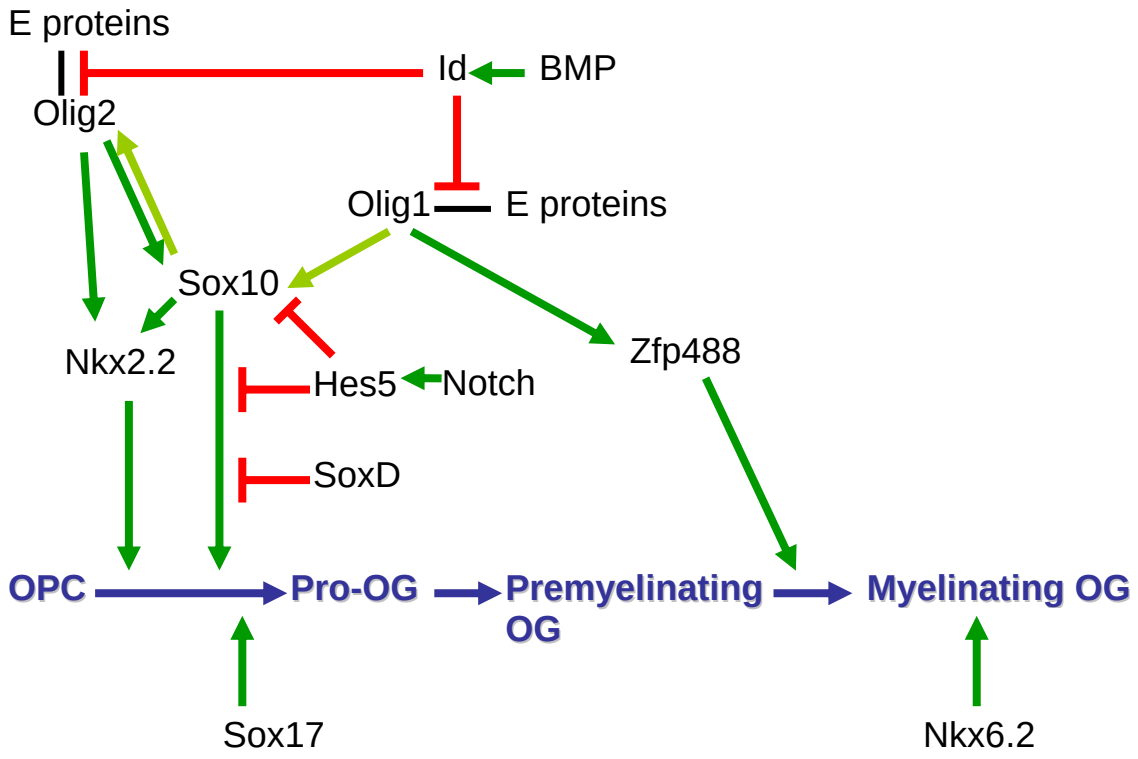


Figure 2.5 Transcriptional network of oligodendrocyte differentiation.

Several TFs (eg. Olig2, Sox10, Olig1, Hes5) and signaling pathways (BMP, Notch) affect OG differentiation. Most of these factors appear to act, in part, via modulating the expression and/or function of other factors. For example, Olig1 may promote OG differentiation by maintaining and inducing the expression of *Sox10* and *Zfp488*, respectively. In contrast, Hes5 may impair this process via inhibiting the expression and/or function of Sox10, which promotes OG differentiation.

Table 2.3 Expression profiles of PDGFαR (♣), PLP/DM20 (♦), and transcription factors implicated in oligodendrocyte development in the central nervous system.

Age	Telencephalon		Diencephalon		Mesen-cephalon	Rhombencephalon		Spinal Cord
	Cortex/ Fiber Tracts	Ganglionic Eminences/ Basal Ganglia	Thalamus	Hypothalamus		Cerebellum	Medulla/ Pons	
E12-12.5	Id2[20],4[9] POU[8] SoxC[6]	Mash1[6] Olig2[33] POU[2] Sox11[6]	Mash1[15] Nkx2.2[23] ♣[24] POU[2]	Ngn3[27] Nkx2.2[23] ♣[24] POU[2]	Id2[20],4[9] POU[8] Sox11[12]	Id2[20]	Brn-1[8] Id2[20],4[9] Ngn3[27] Nkx2.2[23] Olig2[37] ♣[24] Sox11[12]	Hoxa2[21],b4[22] Id4[9] Mash1[15],Ngn3[27] Nkx2.2[21] Olig1,2[41] ♣[24] POU[8] SoxC[12,6],D[29], E[41, 30,31]
E13-13.5	Hes5[5] Hoxa2[39] SCIP[32]	Hes5[5] Hoxa2[39] Mash1[33] Olig1,2[19,33] ♣[34],♦[35] POU[2]	Hoxa2[39] Mash1[33] Nkx2.2[23] Olig1,2[41] ♦[19,35] POU[2]	Hoxa2[39] Mash1[33] Ngn3[27] Nkx2.2[23] Olig1,2[33] ♦[19,35] POU[2]	Hes5[5] Mash1[33] SCIP[32] Sox11[12]	Hes5[5] SCIP[32]	Hes5[5] Ngn3[27] Nkx2.2[37] Olig1,2[41] ♣[24],♦[35] SCIP[32] Sox10[13], 11[12]	Hes5[5] Hoxa2[7] Ngn3[14,27] Nkx2.2[26] Olig1,2[18, 41] ♣[18,41,24] Sox9[31],10[41],11[12] Sox17[28]
E14-14.5	Hes5[1] SoxC[6]	Olig1,2[34] ♣[34] POU[2] Sox10[34]	Hes5[1] Hoxa2[39] Olig1,2[41] ♣[24] POU[2] SoxC[6]	Hes5[1] Ngn3[27] Olig1,2[34] ♣[34],♦[35] POU[2] Sox10[34]	Hes5[1] Sox11[12]	Hes5[1]	Hes5[1] Ngn3[27] Olig1,2[41] Sox11[12]	Hes5[1] Hoxa2[21],b4[22] Ngn3[27,14] Nkx2.2[18,21] Olig1,2[41] ♣[18,41,♦ 38] SoxC[12,6],D[29], E[41,30,31] Zfp488[38]
E15-15.5	Id2[20] POU[8] Sox11[12]	Olig2[19] ♣[19] Sox11[12]	Nkx2.2[23] ♦[35] Sox11[12]	Ngn3[27] Nkx2.2[23] ♦[35] Sox11[12]	♣[24] POU[8,32] Sox11[12]		Ngn3[27] ♣[24],♦[35] POU[8,32] Sox10[13], 11[12]	Brn-1,2[8] Hoxa2[7] Ngn3[27] Nkx2.2[26] Olig1,2[18,33] ♣[18,41,24],♦[18,35] SoxE[41,30,31],11[12]
E16-16.5	♣[25]	POU[2]	♣[25] POU[2]	Ngn3[27] ♣[25],♦[35] POU[2]			♣[25],♦[35] Sox10[40]	Hoxa2[21],b4[22] Nkx2.2[21] Olig2[21] ♦[35] Sox8[30],9[31],17[28],D[29]
E17-17.5	Brn-2[8],SCIP[32] Hes5[1] Id2[20] ♣[34] Sox10[13],C[12,6]	Brn-2[8] ♣[34]	Hes5[1] SoxC[12,6]	Brn-2[8] Ngn3[27] ♦[35] Sox11[12]	SCIP[32] Sox11[12]	Id2[20] Olig2[26] ♣[26] Sox11[12]	Hes5[1] Olig2[26] ♣[26],♦[35] Sox11[12]	Hes5[1] Hoxa2[7] ♣[24],♦[35] Sox9[32],C[6]
E18-18.5	Olig1,2[34] ♣[34] rKr2[16] SCIP[32] Sox10[34]			Nkx6.2[10] ♦[35]	Nkx6.2[10] SCIP[32]		Nkx6.2[10] ♦[35]	Hoxa2[21],b4[22] Nkx2.2[21],6.2[10] Olig2[21] ♣[26],♦[26,35] SoxD[29],E[4,30,31] Zfp488[38]
E19-19.5	Sox11[12]			♦[35]	Sox11[12]	Sox11[12]	♦[35]	Hoxa2[7] ♦[35]
P	Brn-2,SCIP[8] Id2[20,36] MyTI[3], rKr2[16] Olig1,2[33] ♣[25],♦[26,35] SoxE[13],C[6], 17[28] Zfp488[38]	♣[25]	♣[25]	♣[25],♦[35]	♣[25]	Id2[20,36] Nkx6.2[10] Olig1,2[33] ♣[25],♦[35] SoxC[6],E[11] Zfp488[38]	♣[25],♦[35]	Nkx2.2[26] ♣[17],♦[26,35] SoxD[29],8[30],10[17], 17[28] Zfp488[38]

N.B. Although TFs and/or OG markers are expressed in the same CNS region, the expression profiles may or may not overlap. For the purpose of this table information was standardized utilizing the Carnegie (C)/Theiler (T) staging system. Utilizing this staging system the day of vaginal plug is considered E1; therefore, the days were adjusted accordingly so as to have consistent expression data. In cases where the staging information was not given[1,4,10,16,17,20,25,26,29-31,37,38,41] the indicated age in the manuscript was utilized. The E listed is for mice; the corresponding rat E is 1.5 days older. Most references utilized *in situ* hybridization, whereas some references also utilized immunohistochemistry[3,4,7,14,15,16,21,22,28-31,33,37,39].

Abbreviations: POU: Brn-1,2 and SCIP; SoxC: Sox4 and 11; SoxE: Sox8, 9, and 10

References: [1]Akazawa et al. (1992), [2]Alvarez-Bolado et al. (1995), [3]Armstrong et al. (1995), [4]Cai et al. (2005), [5]Casarosa et al. (1999), [6]Cheung et al. (2000), [7]Hao et al. (1999), [8]He et al. (1989), [9]Jen et al. (1996), [10]Komuro et al. (1993), [11]Kordes et al. (2005), [12]Kuhlbrodt et al. (1998a), [13]Kuhlbrodt et al. (1998b), [14]Lee et al. (2003), [15]Lo et al. (1991), [16]Lovas et al. (2001), [17]Lu et al. (2002), [18]Lu et al. (2000), [19]Nery et al. (2001), [20]Neuman et al. (1993), [21]Nicolay et al. (2004a), [22]Nicolay et al. (2004b), [23]Price et al. (1992), [24]Pringle and Richardson (1993), [25]Pringle et al. (1992), [26]Qi et al. (2001), [27]Ravassard et al. (1997), [28]Sohn et al. (2006), [29]Stolt et al. (2006), [30]Stolt et al. (2004), [31]Stolt et al. (2003), [32]Suzuki et al. (1990), [33]Takebayashi et al. (2000), [34]Tekki-Kessaris et al. (2001), [35]Timsit et al. (1995), [36]Tzeng and De Vellis (1998), [37]Vallstedt et al. (2005), [38]Wang et al. (2006), [39]Wolf et al. (2001), [40]Zhou & Anderson (2002), [41]Zhou et al. (2000).

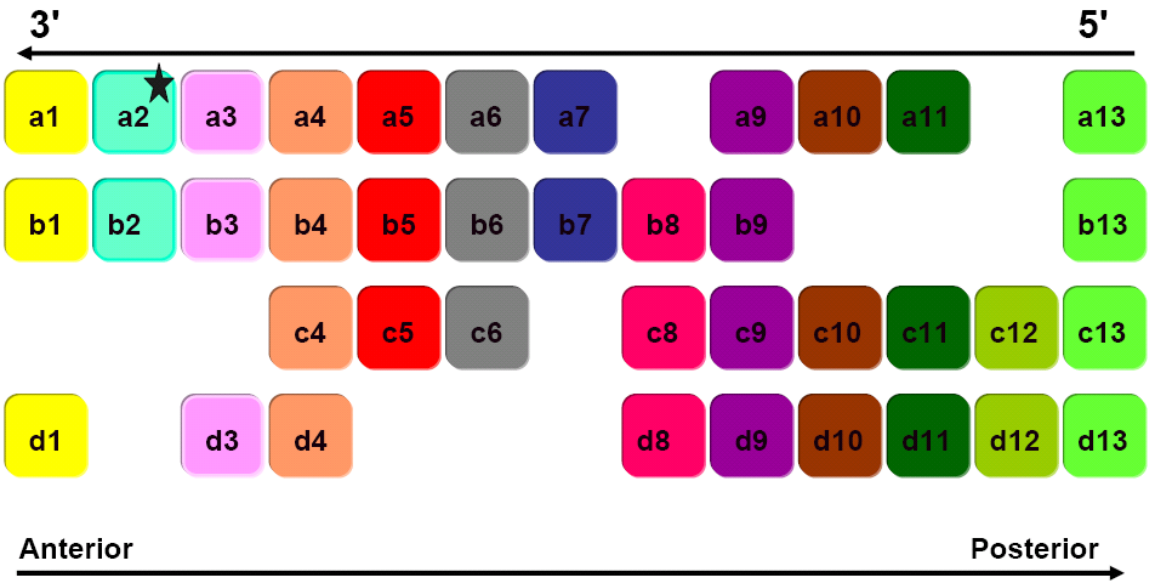


Figure 2.6 There are 39 mouse and human *Hox/HOX* genes which are organized into four clusters located on different chromosomes.

The genes are shown in the order in which they are found on the chromosomes. Genes located at the same position in different chromosomes are known as paralogs. There are 13 paralogous groups in total; however, not all groups contain a full complement of genes. For example, paralog group 2 has only two members: *Hoxa2*, which is the gene our laboratory is interested in (star), and *Hoxb2*. Furthermore, *Hox* genes located at the 3' end of the chromosomes exhibit a more anterior limit of expression than those located at more 5' positions. [Adapted from Scott (1992) and Santagati and Rijli (2003)]

expression with 3′ genes exhibiting more anterior limits than 5′ genes (Duboule and Dollé 1989; Graham et al. 1989). In turn, *Hox* genes located at the same position in different clusters are called paralogs. There are 13 paralogous groups in total; however, not all groups contain a full complement of genes (Scott 1992). Genes within each paralogous group exhibit similarities in sequence (Nazarali et al. 1992; Tan et al. 1992), as well as expression profiles (Gaunt et al. 1989; Hunt et al. 1991).

As our laboratory is interested in elucidating the role of one specific Hox TF, Hoxa2, in OG development, this section of the literature review will describe the expression, regulation, and function of Hoxa2 during embryonic development with specific emphasis on the CNS.

2.2.1 *Hoxa2* mRNA and protein expression

Paralog group 2, which exhibits sequence homology with the *Drosophila* gene *proboscipedia* (Cribbs et al. 1992), consists of only two members: *Hoxa2* and *Hoxb2* (Akin and Nazarali 2005; Hunt et al. 1991; Nazarali et al. 1992). The murine *Hoxa2* gene, which resides on chromosome 6, encodes a 41kD, 372 amino acid protein (Tan et al. 1992). Initially, Northern blot analysis at different embryonic ages found that *Hoxa2* expression was most abundant in 12 dpc mice, progressively decreasing with further embryonic development (Tan et al. 1992). Subsequently, it was found to be expressed in multiple regions including the VII and VIII cranial ganglia (Hunt et al. 1991; Tan et al. 1992), the mesenchyme of the second and third branchial arches (Hunt et al. 1991), as well as the spinal ganglia, larynx, lungs, vertebrae, sternum, intestine (Tan et al. 1992), and CNS (Hao et al. 1999; Nicolay et al. 2004a; Tan et al. 1992; Wolf et al. 2001).

In the CNS, *Hoxa2* mRNA and protein is expressed in the embryonic telencephalon (Tan et al. 1992), diencephalon (Wolf et al. 2001), hindbrain (Davenne et al. 1999; Nonchev et al. 1996; Tan et al. 1992), and spinal cord (Hao et al. 1999; Nicolay et al. 2004a; Tan et al. 1992). In fact, at the 12-somite stage *Hoxa2* is the most anteriorly expressed *Hox* gene with its anterior limit of expression located at the boundary between r1 and r2 (Nonchev et al. 1996). Although it is found in all rhombomeres posterior to this boundary, its expression is higher in r3 and r5 (Hunt et al. 1991; Nonchev et al. 1996). Expression analysis along the dorsal-ventral axis shows that *Hoxa2* is found primarily in the dorsal regions of the hindbrain (Davenne et al. 1999). In comparison, Hoxa2 protein

expression in the spinal cord is evident at E10 in two distinct ventrolateral regions of the neural tube that correspond to the future ventral horns (Hao et al. 1999). In this region Hoxa2 exhibits an overlapping expression profile with Islet1, a marker for MNs. Hence, this suggests MNs express Hoxa2 in the E10 spinal cord. At E14 Hoxa2-immunoreactive cells are also evident in the dorsal horns of the spinal cord, and by E18 more cells are stained in the dorsal horns than in their ventral counterparts (Hao et al. 1999). In the diencephalon, Hoxa2 is expressed in the thalamus and hypothalamus at E12.5 (Wolf et al. 2001). In the telencephalon, it is expressed in the neuroepithelium of the MGE, as well as the dorsal telencephalon at E12.5 and E14 (Tan et al. 1992; Wolf et al. 2001). In glial cultures obtained from the telencephalon Hoxa2 is expressed by glial fibrillary acidic protein $(GFAP)^+$ astrocytes and $O4^+$ pro-OGs (Hao et al. 1999).

2.2.2 Factors regulating *Hoxa2* expression

Multiple factors affect the expression of *Hoxa2*, particularly in the hindbrain [Figure 2.7]. For instance, its lack of expression in r1 appears to be due to the repressive activity of fibroblast growth factor 8 (FGF8), which is a signaling molecule expressed in the organizing center located at the midbrain-hindbrain boundary known as the isthmus (Irving and Mason 2000). In comparison, *Hoxa2* expression in specific rhombomeres posterior to r1 is under the control of several distinct enhancers. For example, its expression in r2 appears to be directed by an enhancer that overlaps with the 3′ end of the *Hoxa2* gene (Frasch et al. 1995). As transfection of *Hoxa2*, along with its co-factor genes *Pbx1a* and *Prep1*, is sufficient to activate a reporter gene driven by this enhancer (Lampe et al. 2004), it suggests that an auto-regulatory loop may be involved in maintaining *Hoxa2* expression in r2. Auto- and cross-regulatory Hox TF loops may also affect *Hoxa2* expression in r4. In particular, researchers have revealed potential Hox/Pbx and Prep/Meis binding sites within the r4 enhancer located in the *Hoxa2* intron. These sites are important for the enhancer's activity since a mutation in one or more of these sites results in a reduction in reporter gene expression in r4. Furthermore, ectopic expression of either *HOXB1* or *Hoxa2* in the hindbrain is able to induce r4 enhancer activity (Tümpel et al. 2007).

An enhancer directing *Hoxa2* expression in r3 and r5 is located in the intergenic region between *Hoxa2* and *Hoxa3* (Nonchev et al. 1996). This enhancer region contains

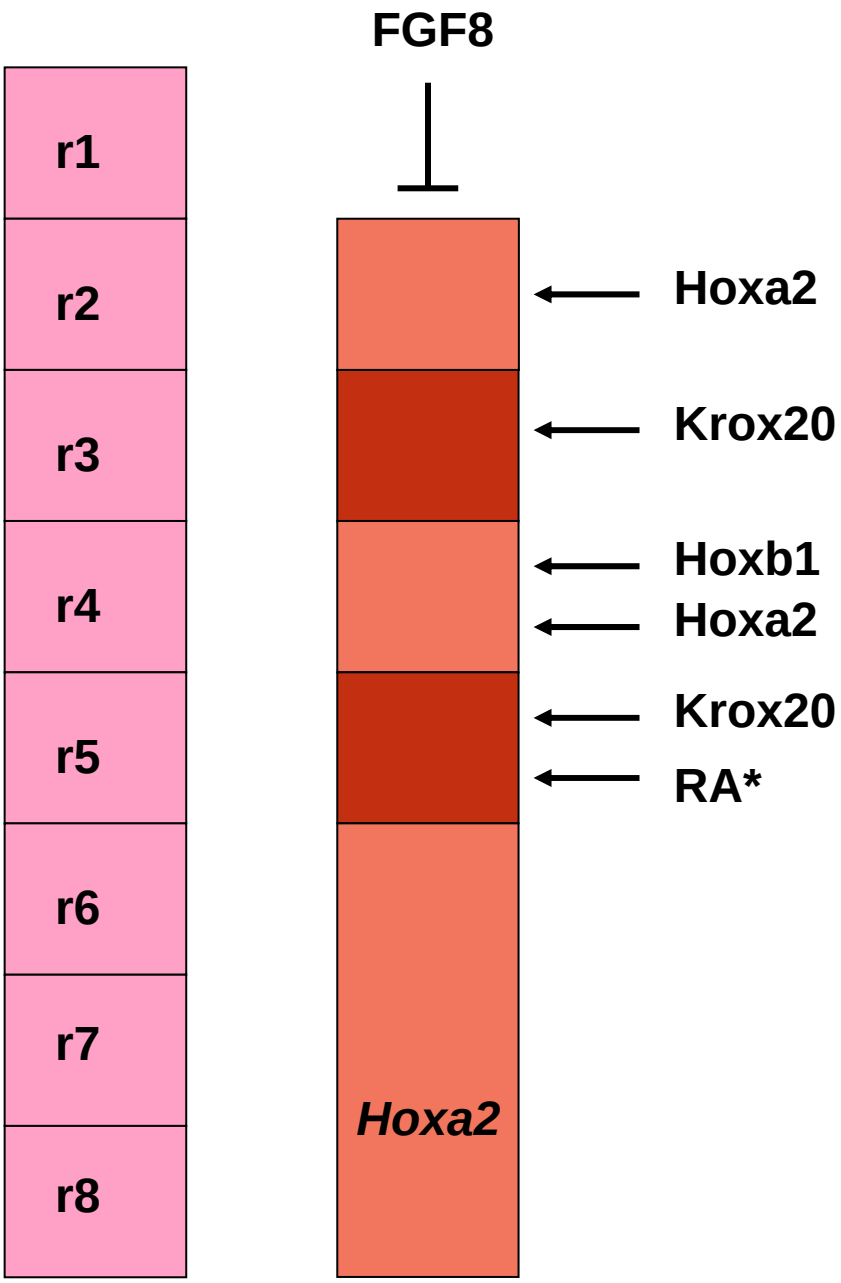


Figure 2.7 *Hoxa2* expression in the hindbrain is regulated by numerous factors.

During early embryonic development the hindbrain is divided into eight units known as rhombomeres (r1 is the most anterior whereas r8 is the most posterior). At the 12-somite stage *Hoxa2* is expressed up to the r1/r2 boundary. Although it is expressed in all rhombomeres posterior to this boundary, it is more intense in r3 and r5 (illustrated by darker orange color). Various factors appear to affect the expression of *Hoxa2* in a rhombomere specific fashion. Please refer to the text [Section 2.2.2, pg. 32] for more details. (*-effect does not appear to be limited to r5)

two Krox20-binding sites. These sites are important for this enhancer activity since reporter gene expression is absent in both rhombomeres in transgenic mice carrying a single point mutation in each of these binding sites (Nonchev et al. 1996). In the absence of *Krox20*, however, the reporter gene expression driven by this enhancer is only reduced or lost in r3 but not in r5 (Maconochie et al. 2001). Although the reason for this disparity is unknown, the mutations in the Krox20-binding sites may affect the ability of other related TFs to bind to the enhancer and, hence, affect r5 expression. Interestingly, recent research suggests that additional elements outside of this enhancer region can impact *Hoxa2* expression in r5. In particular, a mutation in the RA response element (RARE), located 3′ of the *Hoxa1* gene (Frasch et al. 1995; Langston and Gudas 1992), results in a reduction in *Hoxa2*'s expression in r5 (Dupé et al. 1997). RA's ability to enhance *Hoxa2* expression, however, does not appear to be limited to this rhombomere since implantation of RA-soaked beads close to or at the level of the isthmus results in the anterior limit of *Hoxa2*'s expression domain expanding up to and sometimes within the mesencephalon (Plant et al. 2000). Furthermore, *HOXA2* is induced by RA in human embryonal carcinoma cell lines (Simeone et al. 1991).

2.2.3 Developmental abnormalities found in *Hoxa2* mutant mice

Hoxa2-/- mice, which can be identified via the absence of the pinna of the external ear (Rijli et al. 1993), exhibit several distinct defects. Most of the mutant mice display a wide cleft of the secondary palate (Gendron-Maguire et al. 1993; Rijli et al. 1993), as well as a malformation of the tongue (Rijli et al. 1993). These defects may explain why the mutant pups do not suckle and, hence, die within the first day (Gendron-Maguire et al. 1993; Rijli et al. 1993). The mutant mice also exhibit a homeotic transformation of the second arch neural crest-derived skeletal elements into first arch derivatives (Gendron-Maguire et al. 1993; Rijli et al. 1993).

In the CNS, *Hoxa2* mutant mice exhibit anomalies in anterior-posterior patterning. In particular, the absence of *Hoxa2* results in an apparent loss or compromise of the r1/r2 and r2/r3 boundaries, respectively, as well as a posterior expansion of r1 (Gavalas et al. 1997). As the cerebellum appears to be derived from r1 (Wingate and Hatten 1999), it is not surprising that these mutant mice exhibit an enlarged cerebellum at 18.5 dpc (Gavalas et al. 1997). This enlargement, in turn, may be explained by the fact

that the external granule cell layer (EGL) is thickened in specific areas of the cerebellum (Gavalas et al. 1997). Indeed, there is a variable reduction in the size of the cerebellum with a concomitant loss of cells in the EGL following early global over-expression of *Hoxa2* in chick embryos (Eddison et al. 2004).

Hoxa2 mutant mice also display anomalies in TF patterning along the dorsal-ventral axis of the hindbrain. In particular, a ventral shift in the $Pax3^{+}$ expression domain occurs in r2 and r3 in the absence of *Hoxa2*. These mutant mice also display a down-regulation of *Pax6* expression in both of these rhombomeres. However, Hoxb2 appears to partially compensate for Hoxa2 in r3, since these expression anomalies are more severe in *Hoxa2;Hoxb2* double knockout mice (Davenne et al. 1999).

As different types of neuronal precursors arise from distinct dorsal-ventral regions of the neural tube, the patterning defects found in *Hoxa2* mutant mice suggest that the development of certain precursors may be affected. Indeed, researchers have found that Lbx1-expressing somatic sensory interneuron precursors, which arise in dp domains (Gross et al. 2002), are lost in r2 and dramatically reduced in r3 in *Hoxa2* mutant mice (Gaufo et al. 2004). As *Hoxa2* is expressed in the dorsal hindbrain (Davenne et al. 1999), its effect on Lbx1 may be direct, however, further research will be needed to verify this.

Similar to Lbx1, the expression of *Evx1*, which selectively labels interneurons arising from the p0 domain (Moran-Rivard et al. 2001), is completely absent in r2 and partly in r3 of *Hoxa2*-/- mice (Davenne et al. 1999). The reason for the loss of these interneurons is unknown, but it is likely indirect due to the fact that *Hoxa2* is expressed dorsally in the hindbrain (Davenne et al. 1999). However, it does not appear that the anomalies in *Pax3* and *Pax6* expression found in these mutant mice can account for this. In particular, although Pax6 is expressed in the p0 domain (Moran-Rivard et al. 2001), *Evx1* expression is present in Pax6-deficient mice (Burrill et al. 1997) suggesting that it is not required for their development. Similarly, the $Evx1^{+}$ expression domain is not affected in the absence of both *Pax3* and *Pax7* (Mansouri and Gruss 1998). Hence, further research will be needed to elucidate whether the anomalies in dorsal-ventral patterning are responsible for the loss of $Evx1^{+}$ interneurons in *Hoxa2* mutant mice.

Evidence also suggests Hoxa2's potential involvement in MN development; however, its exact role is debatable. In particular, trigeminal branchiomotor neurons,

which arise from the Nkx2.2^{+} pMNv domain (Briscoe et al. 1999), develop in r2 in the absence of *Hoxa2* (Gaufo et al. 2004), therefore, suggesting that it is not required for the development of these neurons. Instead, r2 and r3 trigeminal MNs exhibit errors in axonal pathfinding in the hindbrains of *Hoxa2* mutant mice (Gavalas et al. 1997). However, other researchers have found that the ventral column of *Phox2b* expression, which corresponds to the pMNv domain (Pattyn et al. 1997; Pattyn et al. 2003), exhibits a dorsal expansion in r2 (Davenne et al. 1999). This, in turn, is accompanied by an abnormal distribution of MNs in r2 with their cell bodies being found in more dorsal locations (Davenne et al. 1999). Furthermore, infection of embryonic chick neural tubes with a *Hoxa2*-expressing retrovirus leads to the development of ectopic trigeminal branchiomotoneurons in infected areas of r1 (Jungbluth et al. 1999). Obviously, resolution of this issue will require further research.

2.2.4 Conclusion

Hoxa2 is expressed in multiple regions of the CNS, as well as by several distinct cell types. Currently, research regarding its regulation and function has primarily focused on the hindbrain. In this CNS region, multiple factors regulate *Hoxa2* expression in a rhombomere-specific fashion. In turn, Hoxa2 is important in anterior-posterior and dorsal-ventral patterning, as well as neuronal development in the hindbrain. Further research is needed, however, to elucidate its function at later stages of embryonic development, such as during oligodendrogenesis.

2.3 Introduction to NCAM and oligodendrocyte development

2.3.1 NCAM and its isoforms

NCAM, a cell surface glycoprotein, exists as three prominent protein isoforms (120, 140, 180 kD), encoded by four predominant transcripts arising from alternative splicing and polyadenylation sites of a single gene (Barbas et al. 1988). NCAMs undergo a variety of post-translational modifications, including glycosylation by enzymes, such as sialyltransferase (Breen et al. 1987; Breen and Regan 1988; Finne et al. 1983; Rothbard et al. 1982). As the activity of this enzyme is developmentally regulated (Breen et al. 1987; Breen and Regan 1988), NCAMs can vary in their sialic acid content with the highest evident in PSA-NCAM (Breen et al. 1987; Breen and Regan 1988; Finne et al. 1983; Rothbard et al. 1982). In turn, sialic acid content is inversely related to NCAM

homophilic binding properties, such as adhesion and rate of aggregation (Hoffman and Edelman 1983; Sadoul et al. 1983).

2.3.2 NCAMs and oligodendrocyte development

The stage of development determines the form of NCAM expressed by an oligodendroglial cell. In particular, PSA-NCAM is expressed primarily by $A2B5^+$ OPCs. Subsequently, its expression is dramatically down-regulated such that less than 5% of $O4^+$ pro-OGs express PSA-NCAM (Trotter et al. 1989). Instead, with continued development oligodendroglial cells express predominantly NCAM-120 (Bhat and Silberberg 1988; Trotter et al. 1989) and to a lesser extent NCAM-140 (Trotter et al. 1989).

Expression profiles of PSA-NCAM and NCAM suggest that they could play distinct roles during OG development. PSA-NCAM's expression profile, as well as its anti-adhesive properties suggests that it could play a role in OPC migration. Indeed, removing the polysialic acid units from the NCAM molecule, using the enzyme endoneuraminidase (endoN), in explant cultures blocks OPC migration from the explant (Wang et al. 1994). Further clarification of its role in OPC migration comes from a study by Barral-Moran and colleagues who found that after the removal of polysialic acid units the cells are still motile but exhibit aberrant movements (Barral-Moran et al. 2003). More recent research suggests that PSA-NCAM is important in PDGF-directed migration of oligodendroglial cells (Zhang et al. 2004).

Research also suggests that PSA-NCAM could play a role in inhibiting OG differentiation since endoN treatment increases the number of $O4^+$ cells, particularly those maintained in a serum free medium (Decker et al. 2000). In addition, PSA-NCAM appears to indirectly affect myelination. Axonal PSA-NCAM expression has been shown to decrease with a time frame that parallels myelination (Nait-Oumesmar et al. 1995). In turn, researchers have found that endoN treatment *in vitro*, as well as *in vivo* results in an increase in the number of myelinated internodes (Charles et al. 2000).

In comparison to its embryonic form, the expression and adhesive properties of NCAM suggest that it could also be involved in myelination. Although this has not been examined directly there is some circumstantial evidence to support this. In particular,

growing oligodendroglial cells on a NCAM substrate results in an increase in the cells' surface areas compared to control (Gard et al. 1996).

2.3.3 Conclusion

Various isoforms of the cell surface glycoprotein NCAM are expressed by oligodendroglial cells *in vitro*. However, current knowledge regarding the function of NCAM is limited to the embryonic isoform, PSA-NCAM.

2.4 Introduction to retinoic acid and oligodendrocyte development

Over the last 15 years there has been a wealth of information reported in the literature regarding OG development and RA, which is an active metabolite of vitamin A. Therefore, the purpose of this section of the literature review is to highlight what is currently known about RA signaling in oligodendroglial cells, as well as its potential role(s) in OG specification and differentiation.

2.4.1 Retinoic acid signaling during oligodendrocyte development

In order for RA to play a role in oligodendrogenesis it has to be synthesized either by the oligodendroglial cells themselves or by cells/tissue within their vicinity. RA synthesis is a two step enzymatic process (Maden 2002) during which retinol (vitamin A) is first converted to retinal by retinol/alcohol dehydrogenases and then, subsequently, to RA via retinaldehyde dehydrogenases (Raldhs). Current information regarding the expression profiles of Raldhs suggests that the ability of oligodendroglial cells to synthesize RA is stage-dependent. The three currently known Raldhs— *Raldh1*, Raldh2, and *Raldh3*— are expressed in specific regions of the CNS (Berggren et al. 1999; Li et al. 2000; Niederreither et al. 2002). Although these regions do not correspond to the neuroepithelial domains from which OPCs are known to arise (Cai et al. 2005; Fu et al. 2002; Kessaris et al. 2006), they are within their vicinity. For example, in the embryonic chick spinal cord Raldh2 is expressed by MNs in the ventral horns from stages 17 to 29. In addition, intense Raldh2 expression is found in the RP of the spinal cord from stage 16 through to stage 27, as well as in the meninges (Berggren et al. 1999). As Raldh2 is the only Raldh known to be expressed in this CNS region, these results suggest that OPCs, which arise in the embryonic chick spinal cord at stage 26 (Novitch et al. 2001), are unable to synthesize RA themselves and, hence, must rely on its secretion by other cells, such as MNs. In contrast to OPCs, evidence suggests that differentiated OGs can

synthesize RA. Raldh2 is expressed by CNP^+ OGs in the adult rat spinal cord (Mey et al. 2005). Furthermore, when the OG cell line OLN-93 is directed to differentiate it also expresses Raldh2 and can synthesize RA in the presence of retinal (Mey and Hammelmann 2000).

RA exists as two isomers, all-*trans* and 9-*cis*, both of which affect OG development *in vitro* (Barres et al. 1994; Laeng et al. 1994; Noll and Miller 1994; Pombo et al. 1999). However, little is known regarding which isomer(s) oligodendroglial cells are exposed to during their development *in vivo*. The only research group that has tried to address this issue is Mey and Hammelman (2000) who found that differentiated OLN-93 cells are only able to synthesize all-*trans* RA. As it is not known how this relates to OG development *in vivo*, further research will be required.

In addition to its synthesis, the level of RA in a cell is also affected by its metabolism. RA is metabolized to 4-oxo RA and 4-hydroxy RA by CYP26 (White et al. 1996). Although CYP26 is expressed by OLN-93 cells (Mey and Henkes 2002), it is not known whether it is expressed by primary oligodendroglial cells. Hence, more research is needed to determine if the ability of oligodendroglial cells to metabolize RA is also stage-dependent.

In order to affect OG development RA must enter the oligodendroglial cell's nucleus and bind to RA receptors (RARs) and retinoid X receptors (RXRs), which exist as multiple isoforms. 9-*cis* RA can bind to both RARs and RXRs, whereas all-*trans* RA can only bind to the former (Allenby et al. 1993). These receptors, in turn, bind primarily as heterodimers, to cis-acting RAREs on DNA and, hence, can alter the expression of downstream effector genes. However, little information is known regarding RAR and RXR expression by oligodendroglial cells. For instance, *RARα* is the only receptor currently known to be expressed by OPCs (Laeng et al. 1994). In addition, the only receptors that are known to be expressed by CNP^+ OGs in the adult rat spinal cord are RARα, RXRα, and RXRγ (Schrage et al. 2006). OLN-93 cells are known to express *RARα*, *RARβ*, and *RARγ*, as well as *RXRα* and *RXRβ* (Mey and Henkes 2002). However, a more detailed expression analysis is needed to determine whether the complement of receptors changes as the oligodendroglial cells progress from precursors to mature myelinating cells.

2.4.2 Retinoic acid and oligodendrocyte specification

Although RA's role in OG specification has not been addressed directly, research suggests that it could be involved in this process, at least in the spinal cord. In the spinal cord, PDGFαR$^+$ OPCs initially arise in a distinct region of the embryonic vVZ (Sun et al. 1998). This region is known as the pMN domain, since it gives rise to MNs at earlier stages of embryonic development (Fu et al. 2002). In turn, the pMN domain, which is specifically demarcated by the expression of the bHLH TF Olig2, is one of five ventral progenitor domains formed in response to graded Shh concentrations (Briscoe et al. 2000; Gritli-Linde et al. 2001). In particular, Shh, which is expressed and secreted by cells of the notochord and FP (Echelard et al. 1993; Marti et al. 1995), initially regulates the expression of the TFs that define the boundaries of these progenitor domains (Briscoe et al. 2000; Ericson et al. 1997; Lu et al. 2000; Pierani et al. 1999). This includes regulating the expression of Nkx2.2 and Pax6 (Ericson et al. 1997), as well as *Olig2* (Lu et al. 2000) and *Irx3* (Briscoe et al. 2000), which define the ventral (Briscoe et al. 2000) and dorsal (Novitch et al. 2001) boundaries, respectively, of the pMN domain.

Interestingly, researchers have found that Shh alone cannot fully recapitulate the TF expression profiles found in the pMN domain. In particular, Nkx2.2 expression, which is excluded from this region during neurogenesis (Fu et al. 2002), is found in neural plate explants treated with Shh concentrations known to give rise to predominantly MNs (Ericson et al. 1997). As Nkx2.2 represses *Olig2* expression at early stages of spinal cord development (Novitch et al. 2001; Zhou et al. 2001), an additional factor must be required to ensure proper establishment of the pMN domain. Recent research has suggested that this factor could be RA since it blocks Shh-induced Nkx2.2 expression in neural plate explants (Novitch et al. 2003). How it is able to accomplish this is uncertain but could involve its ability to enhance the expression of Pax6 (Novitch et al. 2003), which maintains and refines the ventral boundary of the pMN domain through cross-repressive interactions with Nkx2.2 (Briscoe et al. 2000). Hence, RA appears to collaborate with Shh to ensure the correct patterning of the pMN domain. Indeed, Shh's ability to induce Olig2 expression is dramatically reduced when retinoid signaling is blocked (Novitch et al. 2003). As Olig2 is critical for OG development in the spinal cord (Lu et al. 2002), RA may be required for this process to occur normally.

In addition to ensuring the proper patterning of the pMN domain, evidence also exists suggesting that RA could be involved in the switch from neurogenesis to gliogenesis in the spinal cord. In particular, activation of two different RAR isoforms, RARα and RARβ, results in most neural progenitor cells differentiating into glial cells or MNs, respectively (Goncalves et al. 2005). Hence, OG specification in the ventral spinal cord could be dependent upon changes in the expression profiles of RARs in the pMN domain. Circumstantial support for this statement comes from the finding that OPCs express *RARα* but not *RARβ* (Laeng et al., 1994).

2.4.3 Retinoic acid and oligodendrocyte differentiation

Research examining RA's role in OG differentiation suggests that it can inhibit (Laeng et al. 1994; Noll and Miller 1994), as well as promote (Barres et al. 1994; Givogri et al. 2001; Pombo et al. 1999) this process. Although the reason for these differential effects is currently unknown, there is evidence to suggest that it could depend on the stage of oligodendrogenesis, the RA concentration, and/or the receptor. In particular, RA treatment of spinal cord cultures, obtained from either E14, E16, or E18 rats, inhibits OG differentiation with the effect being most evident in cultures obtained from the E14 rats (Noll and Miller 1994). As $O4^+$ pro-OGs and $GalC^+$ pre-myelinating OGs are known to first arise in the spinal cord at E16 and E18, respectively (Fok-Seang and Miller 1994), it could be postulated that RA inhibits OG differentiation at the OPC stage. This, in turn, may explain why Laeng et al. (1994) and Givogri et al. (2001) report opposing research findings. Both of these research groups used cultures obtained from the cerebral hemispheres of neonatal rats. However, as Laeng et al (1994) purified their cultures for OPCs prior to RA treatment their cells would be expected to be more immature than those used by Givogri et al. (2001). Alternatively, the contradictory findings could reflect a concentration-dependent effect since these two groups also utilized different RA concentrations. There is also evidence to suggest that RA's effect on OG differentiation could be receptor dependent. This is due to the fact that although all-*trans* RA enhances *MBP* expression (Givogri et al. 2001), it is unable to induce MBP promoter activity in C6 cells co-transfected with *RARα* and the MBP-promoter construct (Pombo et al. 1999). Hence, different receptors could be responsible for RA's opposing effects on OG differentiation.

How RA is able to inhibit OG differentiation is unknown but it may involve its ability to enhance the expression of PSA-NCAM (Husmann et al. 1989), which is implicated in inhibiting OG differentiation (Decker et al. 2000). In particular, in carcinoma cell lines, RA enhances the activity of sialyltransferase, which adds sialic acid residues to the NCAM protein, in a concentration-dependent manner (Deutsch and Lotan 1983). As this effect is first evident one day after RA treatment (Deutsch and Lotan 1983), downstream targets of RA, such as Hox and Krox TFs (Conlon and Rossant 1992), may be involved. In turn, Hox TFs have been implicated in the regulation of NCAM expression (Jones et al. 1992). Furthermore, Hoxa2 (Hao et al. 1999; Nicolay et al. 2004a) [Section 3.3.1, pg. 52] and Hoxb4 (Nicolay et al. 2004b) [Section 4.3, pg. 72] have been shown to be expressed by cells of the OG lineage. Therefore, RA may inhibit OG differentiation via enhancing the expression of PSA-NCAM, potentially with the aid of downstream effectors, such as Hox TFs.

In comparison, there could be multiple mechanisms by which RA promotes OG differentiation. For example, in the oligodendroglial cell line OLN-93, RA enhances the expression of leukemia inhibitory factor (*LIF*) (Mey and Henkes 2002). LIF, in turn, promotes terminal differentiation of purified OPCs when it is added daily for six days at a concentration of 4 ng/ml (Ibarrola et al. 1996). RA also enhances the expression of the myelin protein genes *PLP* (López-Barahona et al. 1993) and *MBP* (Givogri et al. 2001; Pombo et al. 1999). Interestingly, the mechanism by which it brings about these effects appears to be different. Whereas RA enhances the activity of the MBP promoter (Pombo et al. 1999), it increases *PLP* expression via enhancing *PLP*'s mRNA stability, which results in an increase in *PLP*'s half life (López-Barahona et al. 1993). Another potential mechanism by which RA could promote differentiation is via raising the intracellular pH (pHi) of oligodendroglial cells (Bernard et al. 2006), which increases with OG differentiation (Boussouf and Gaillard 2000). The level of OG differentiation is dependent on the pHi, with the highest level of differentiation evident around pHi 7.15 (Bernard et al. 2006). RA could also promote OG differentiation via affecting the expression of cell cycle regulators. For instance, in the presence of RA, the RAR can repress the promoter activity of the TF E2F-1 (Nygård et al. 2003), which induces the expression of genes involved in DNA replication or mitosis (Polager et al. 2002). This

repression, in turn, could lead to cell cycle withdrawal and, subsequently, OG maturation. RA's ability to promote differentiation also appears to depend upon members of the p53 TF family since RA-induced differentiation is blocked when purified OPCs are infected with dominant-negative forms of p53 or p73 (Billon et al. 2004). How p53 and p73 promote OG differentiation is not known, but it may involve their ability to induce the gene expression of two cyclin-dependent kinase inhibitors, *p21* and *p27* (Fontemaggi et al. 2002). Accordingly, over-expressing *p27* in CG4 cells enhances MBP promoter activity (Wei et al. 2003; Wei et al. 2004) via increasing Sp1 binding to the MBP promoter (Wei et al. 2003). In comparison, there is a dramatic decrease in MBP expression in the cerebellum of *p21*-/- mice at P4 and P6 (Zezula et al. 2001).

2.4.4 Conclusion

Oligodendroglial cells express various components of the RA signaling pathway; however, further research is needed to determine if the components of this pathway change as the cells progress from precursors to mature myelinating cells. Current evidence suggests that RA plays multiple roles in OG development. However, as most of this information was obtained utilizing *in vitro* assays, it will need to be confirmed *in vivo*.

Preamble to Chapter 3

The objectives of this chapter are to examine the expression of Hoxa2 during the early stages of OG development, as well as to determine whether Hoxa2 is required for the specification and/or early maturation of OGs.

(Manuscript published, Appendix C)

Nicolay DJ, Doucette JR, Nazarali AJ. 2004a. Early stages of oligodendrocyte development in the embryonic murine spinal cord proceed normally in the absence of *Hoxa2*. Glia 48:14-26.

3.0 EARLY STAGES OF OLIGODENDROCYTE DEVELOPMENT IN THE EMBRYONIC MURINE SPINAL CORD PROCEED NORMALLY IN THE ABSENCE OF *Hoxa2* (Manuscript published).

3.1 Summary

Recent discoveries have enhanced our knowledge of the transcriptional control of OG development. In particular, the TFs Olig2, Pax6, and Nkx2.2 have been shown to be important in the specification and/or maturation of the OG lineage. Although numerous other TFs are expressed by OGs, little is known regarding their role(s) in oligodendrogenesis. One such TF is Hoxa2, which was recently shown to be expressed by $O4^+$ pro-OGs. The objectives of this study are to examine the expression of Hoxa2 during the early stages of OG development, as well as to determine whether Hoxa2 is required for specification and/or early maturation of OGs. Immunocytochemical analysis of primary mixed glial cultures demonstrated that Hoxa2 is expressed throughout oligodendrogenesis, diminishing only with the acquisition of a myelinating phenotype. Serial transverse spinal cord sections from E12.5, E14.25, E16, and E18 *Hoxa2*+/+, *Hoxa2*+/-, and *Hoxa2*-/- mice were subjected to single and double immunohistochemical analysis in order to examine Hoxa2, Olig2, Nkx2.2, and Pax6 expression profiles. Results obtained from *Hoxa2*+/+ and *Hoxa2*+/- mice suggest that Hoxa2 is expressed by migratory oligodendroglial cells. In addition, comparison of spinal cord sections obtained from *Hoxa2*+/+, *Hoxa2*+/-, and *Hoxa2*-/- mice suggest that specification and early maturation of OGs proceeds normally in the absence of *Hoxa2*, since there are no obvious alterations in the expression patterns of Olig2, Nkx2.2, and/or Pax6. Hence, although Hoxa2 is expressed throughout OG development, it does not appear to be critical for early stages of oligodendrogenesis in the murine spinal cord.

3.2 Introduction

OPCs, which are characterized by *PDGFαR* expression, first appear in the murine spinal cord at E12.5 in a distinct region of the vVZ (Sun et al. 1998). This region has been shown to correspond to the pMN domain, which is one of five ventral progenitor domains formed in response to graded Shh concentrations (Briscoe et al. 2000; Ericson et al. 1997; Fu et al. 2002; Novitch et al. 2001). In particular, Shh, which is expressed and secreted by cells of the notochord and FP, initially regulates the expression of the TFs

that define the ventral (Nkx2.2/Pax6) and dorsal (*Olig2*/*Irx3*) boundaries of the pMN domain (Briscoe et al. 2000; Bumcrot et al. 1995; Echelard et al. 1993; Ericson et al. 1997; Marti et al. 1995; Novitch et al. 2001) [Figure 3.1]. Subsequently, repressive interactions between these TFs maintain and refine these boundaries (Briscoe et al. 2000; Novitch et al. 2001).

Olig2, Pax6, and Nkx2.2 have been shown to be important in OG specification and/or maturation, since mutations of these genes lead to anomalies in these processes (Lu et al. 2002; Qi et al. 2001; Sun et al. 1998). Olig2, which specifically demarcates the pMN domain and is expressed throughout OG development, is critical for spinal cord oligodendrogenesis since, in its absence, expression of *PDGFαR*, *Sox10*, *MBP*, and *PLP/DM20* are undetectable (Lu et al. 2002). Mice deficient in Pax6 exhibit a delay of 1 day and a dorsal shift in the appearance of $PDGF\alpha R^+$ cells (Sun et al. 1998). Nkx2.2 is important in the spatial aspect of $PDGF\alpha R^+$ OPC specification, as demonstrated by a ventral expansion, as well as an increased number of $Olig2^+$ cells (Qi et al. 2001). It has also been hypothesized that a separate OPC lineage arises from the $Nkx2.2^+$ p3 domain. In addition, Nkx2.2 is important in OG maturation due to the fact that *Nkx2.2* mutant mice display not only a delay and reduction in *MBP* and *PLP/DM20* expression in the white matter but also an absence of these markers in the gray matter (Qi et al. 2001).

Although numerous other TFs are expressed by cells of the OG lineage, researchers have been unable to define where they ultimately lay in the transcriptional control of oligodendrogenesis. One TF that has been shown to be expressed by $O4^+$ pro-OGs is Hoxa2 (Hao et al. 1999). *Hoxa2* is one of 39 mouse and human *Hox/HOX* genes that are organized into four clusters (*Hox* A, B, C, D) located on different chromosomes (Favier and Dollé 1997; Santagati and Rijli 2003; Scott 1992). These genes are characterized by a 180 base pair homeobox, which encodes a 60 amino acid homeodomain (McGinnis et al. 1984a; McGinnis et al. 1984b). Hox TFs can regulate the expression of downstream effector genes by binding to specific nucleotide sequences through the homeodomain (Hirsch et al. 1990; Hoey and Levine 1988; Jones et al. 1993; Jones et al. 1992). We have further investigated Hoxa2 expression, as well as its potential role(s) in OG development. Our results suggest that although Hoxa2 is express-

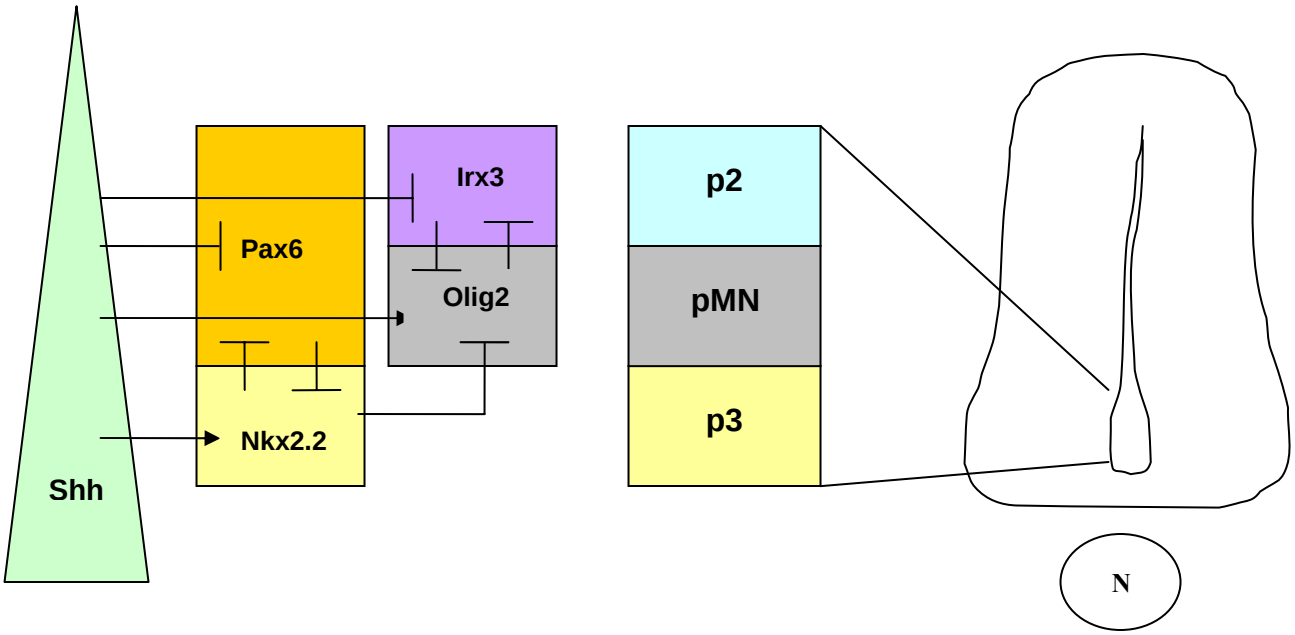


Figure 3.1 PDGFαR$^+$ oligodendrocyte precursor cells originate from the pMN domain.

Shh, which appears to exhibit a ventral to dorsal decreasing concentration gradient, represses (⊥) and enhances (→) the expression of class I (Pax6 and *Irx3*) and II (Nkx2.2 and *Olig2*) TFs, respectively, that define the boundaries of the pMN domain. These boundaries are further maintained and refined by repressive interactions between these TFs (Briscoe et al. 2000; Echelard et al. 1993; Ericson et al. 1997; Fu et al. 2002; Novitch et al. 2001; Timsit et al. 1995). N, Notochord.

ed by cells of the OG lineage, it does not appear to be critical for the specification or early maturation of these cells in the murine spinal cord.

3.3 Materials and Methods

3.3.1 Primary glial cultures and immunocytochemistry

Cerebral hemispheres from newborn mice were dissected in Dulbecco's modified Eagle's medium/ F12/10% fetal bovine serum (DMEM/F12/10% FBS). Subsequently, single cell suspensions, obtained by forcing diced tissue through a 75 μm Nitex mesh, were plated in DMEM/F12/10% FBS for 4 days to allow adherence of the cells. They were then cultured for two 1-week periods, respectively, in a B104 conditioned medium and a low serum-containing (0.3%) growth medium (GP medium) [described previously by Gard and Pfeiffer (1990) and Doucette and Devon (1994)] supplemented with 10 ng/ml basic FGF. Culture medium was changed every 4 days.

The following primary antibodies were used for immunocytochemical detection: anti-Hoxa2 (1:800; Hao et al. 1999), A2B5 (1:10, hybridoma; American Type Cell Culture [ATCC]), O4 (1:10, hybridoma; Sommer and Schachner 1981), anti-GalC (1:20, hybridoma; Ranscht et al. 1982), and anti-MBP (1:800; Sternberger Monoclonals, Lutherville, MD). A2B5, O4, GalC, and MBP are used to mark cells at different stages of OG development [Figure 3.2]. In particular, the monoclonal antibody A2B5 is used to recognize a complex ganglioside expressed on the surface of OPCs (Abney et al. 1983; Eisenbarth et al. 1979). The monoclonal antibody O4 recognizes surface epitopes expressed by pro-OGs (Bansal et al. 1989). GalC, the major galactosphingolipid of myelin, marks pre-myelinating OGs (Ranscht et al. 1982). Finally, expression of myelin proteins, such as MBP, marks mature myelinating OGs. Double labeling cells for Hoxa2 and A2B5, O4, or GalC was conducted using a procedure previously described by Doucette and Devon (1994) with the following modifications: live cells were incubated for 30 min at 37°C in A2B5, O4, or anti-GalC monoclonal antibodies diluted in phosphate-buffered saline (PBS); the blocking and permeabilization steps were combined with a 30-min incubation in 3% skim milk powder solution containing 0.1% Triton X (3% SM/0.1% TX); and cells were incubated for 4 h at room temperature (RT) in anti-Hoxa2 diluted in 1% SM/0.03% TX. The secondary antibodies used included goat anti-mouse IgM fluorescein isothiocyanate (FITC) (1:100, A2B5/ O4; Sigma, Oakville, ON),

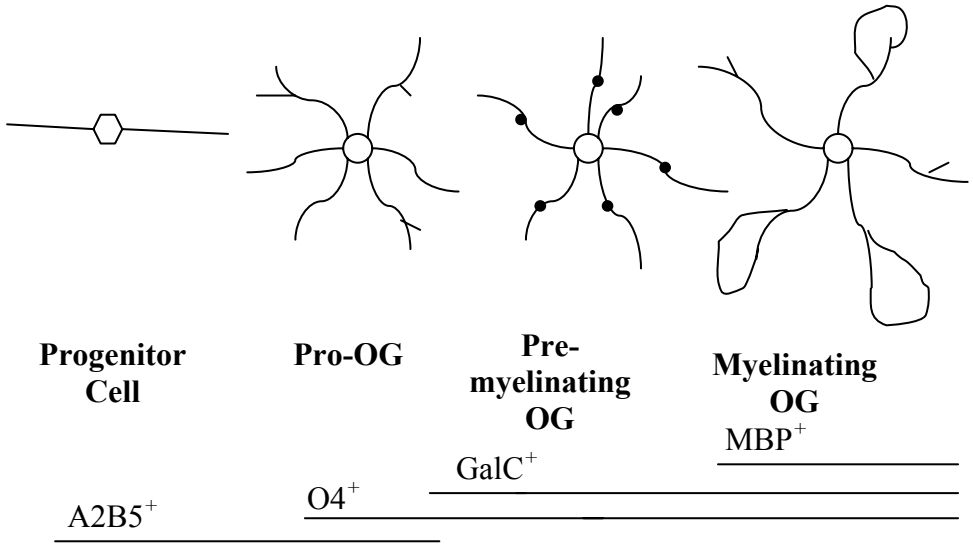


Figure 3.2 Oligodendrocytes progress through four distinct stages *in vitro*, characterized by unique morphological and antigenic phenotypes (Abney et al. 1983; Asou et al. 1995; Bansal et al. 1989; Duchala et al. 1995; Fok-Seang and Miller 1994; Miller 1996; Noble et al. 1988; Raff et al. 1985; Raff et al. 1983; Sommer and Schachner 1981).

donkey anti-mouse IgG FITC (1:50, GalC; BIO/CAN Scientific, Mississauga, ON), and goat anti-rabbit IgG CY3 (1:200, Hoxa2; BIO/CAN Scientific). Between incubations, cultures were washed twice for 5 min in PBS. Double immunostaining Hoxa2 with MBP was done using a procedure previously described by Doucette and Devon (1994), with two modifications: (1) cells were incubated in anti-MBP and then anti-Hoxa2 for 30 min and 4 h, respectively; (2) between anti-MBP and anti-Hoxa2 incubations, cells were rinsed in PBS and were then incubated for 30 min in 3% SM/0.1% TX. The nuclei in all cell cultures were stained using Hoechst dye (Sigma, ON). The percentage of OGs ($O4^+$, $GalC^+$, and MBP^+ cells) that expressed Hoxa2 was calculated from 2 coverslips in which 100–200 cells were counted.

3.3.2 *Hoxa2* transgenic mice and immunohistochemistry

Hoxa2+/+, *Hoxa2*+/-, and *Hoxa2*-/- C57 black mice (Gendron-Maguire et al. 1993) were obtained by timed heterozygous matings. They were staged according to the Carnegie (Butler and Juurlink 1987) and Theiler (1972) staging systems [Appendix A]. The E and corresponding C/T stages used were as follows: E12.5 (C16/T19), E14.25 (C19+/T21), E16 (C23/T23), and E18 (T25); the Carnegie staging system does not go beyond stage 23. Although *Hoxa2*-/- mice could be identified by pinna deformities at later stages of embryonic development (i.e., E14+), genotypes were confirmed by polymerase chain reaction (PCR) analysis (Gendron-Maguire et al. 1993).

Mouse embryos were fixed by immersion in 4% formaldehyde at 4°C for 24–48 h depending on their age. Embryos were then rinsed and stored in 20% sucrose until they were processed. Serial cryostat sections (8 μm) were collected on gelatin-coated coverslips and allowed to dry for ~1 h prior to staining. The following primary antibodies were used: anti-Olig2 [gift of Dr. Hirohide Takebayashi (Takebayashi et al. 2000); E12.5, 1:4,000; rest, 1:2,000], anti-Nkx2.2 (hybridoma; Developmental Studies Hybridoma Bank; E12.5, 1:4; rest, 1:2), anti-Hoxa2 (Hao et al. 1999; E12.5, 1:3,000; rest, 1:1,500), and anti-Pax6 (Developmental Studies Hybridoma Bank; E12.5, 1:1,000; rest, 1:500). Immunohistochemical analysis of Hoxa2 and Olig2 was performed with a procedure previously described by Hao et al. (1999). The following modifications were made with regard to time intervals: PBS washes were 8 min instead of 10 min; 3%

SM/0.1% TX blocks/permeabilizations were 20 min versus 30–60 min; and incubation in the secondary antibody was 30 min versus 45 min.

Immunohistochemical analysis of Nkx2.2 and Pax6 used a modified Mouse on Mouse (MOM)™ (Vector Laboratories, Burlingame, CA) staining procedure. Briefly, sections were washed twice in PBS for 8 min, followed by a 20-min permeabilizing/blocking period in 3% SM/0.1% TX. After two 2-min washes in PBS, sections were incubated for 1 h in MOM Mouse Ig blocking solution. Following a 5-min block in MOM diluent, sections were incubated at RT for 30 min and 10 min in primary and secondary antibodies, respectively. Two 2-min PBS washes were done between incubations. Endogenous peroxidase activity was then blocked by a 8-min incubation in a 0.3% hydrogen peroxide solution. After two 4-min PBS washes, sections were incubated in avidin-biotin complex (Vectastain® Elite ABC; Vector) for 20 min at RT. The sections were subsequently washed for 8 min in PBS and then 0.175 M sodium acetate, respectively. For diaminobenzidine tetrahydrochloride (DAB) staining, a 2-min incubation was performed as described previously by Hao et al. (1999). Sections were then washed in PBS, dehydrated, and mounted in Permount® (Fisher Scientific, Nepean, ON).

For immunofluorescent double labeling, sections were washed twice in PBS for 8 min, followed by a 20-min block/permeabilization in 3% SM/0.1% TX. They were then incubated overnight in primary antibodies [anti-Hoxa2 (1:200), anti-Olig2 (1:2,000), anti-Pax6 (1:100), anti-Nkx2.2 (concentrate), anti-Islet1 (Developmental Studies Hybridoma Bank, 1:3,000), and anti-β-tubulin (1:8,000)] diluted in 1% SM/0.03% TX. After two 8-min PBS washes, sections were incubated for 3 h in Alexa 594 and 488 secondary antibodies (Molecular Probes, Eugene, OR). Finally, sections were rinsed in PBS and were mounted in Citifluor® (Merivac, Montreal, QC). (When sections were stained for β-tubulin an additional 8-min incubation in Hoechst dye was performed to identify nuclei.)

3.4 Results

3.4.1 Hoxa2 is expressed throughout oligodendrocyte development *in vitro*

Immunocytochemical analysis of primary mixed neopallial glial cell cultures

demonstrates that Hoxa2 is expressed throughout OG development, diminishing only with the acquisition of a myelinating phenotype [Figure 3.3]. In particular, greater than 95% of $O4^+$ pro-OGs and $GalC^+$ pre-myelinating OGs express Hoxa2 [Figure 3.3E,H]. As OGs mature to the MBP^+ myelinating phenotype, the percentage of Hoxa2-expressing cells drops to 56% [Figure 3.3K]. The location of immunoreactivity is primarily nuclear; however, at the pre-myelinating stage cytoplasmic staining is also observed. Although Hoxa2 is expressed in most cells in the OG lineage, its expression in these mixed glial cell cultures is not limited to OGs. This is demonstrated by the expression of Hoxa2 in cells that are A2B5, O4, GalC, and MBP-negative [Figure 3.3B,E,H,K].

3.4.2 *Hoxa2* expression is not required for oligodendrocyte specification in the murine spinal cord

At E12.5 (C16/T19) Hoxa2 is expressed predominantly in the ventral horns [Figure 3.4A]. In contrast, Nkx2.2 and Olig2, which may mark separate OPC lineages, are expressed in distinct regions of the vVZ in *Hoxa2*+/+ and *Hoxa2*+/- mice [Figure 3.4B,C]. In particular, Nkx2.2 is expressed in the p3 domain, which is adjacent to the FP. Olig2 is expressed just dorsal to the $Nkx2.2^+$ p3 domain in the pMN domain. Although Olig2 expression is restricted to the vVZ, some $Nkx2.2^+$ cells are found in the adjacent mantle layer [Figure 3.4B]. Double immunofluorescent staining demonstrated that some of the $Nkx2.2^+$ cells located in the mantle layer co-express Hoxa2 [Appendix B]. Many $Islet1^+$ MNs in the mantle layer also co-express Hoxa2 [Figure 3.4I,J].

At E14.25 (C19+/T21), Hoxa2 is expressed throughout the mantle layer [Figure 3.4E]. Although at E14.25, Nkx2.2 and Olig2 continue to be expressed in the vVZ, $Nkx2.2^+$ and $Olig2^+$ cells are also found in the mantle layer [Figure 3.4F,G]. Occasionally, Olig2-immunoreactive cells are observed in the marginal layer at this stage of development [Appendix B]. Double immunofluorescent staining demonstrated that few if any cells co-express Nkx2.2 and Olig2 at E14.25 (C19+/T21) [Appendix B]. However, most $Nkx2.2^+$ cells located in the mantle layer express Hoxa2 [Figure 3.4K,L]. The absence of Hoxa2 expression in the vVZ during OG specification would suggest that it may not be critical for this process in the spinal cord. To test this directly, immunohistochemical analysis of *Hoxa2*+/+, *Hoxa2*+/-, and *Hoxa2*-/- spinal cord sections were conducted to determine whether Nkx2.2, Pax6, and/or Olig2 expression

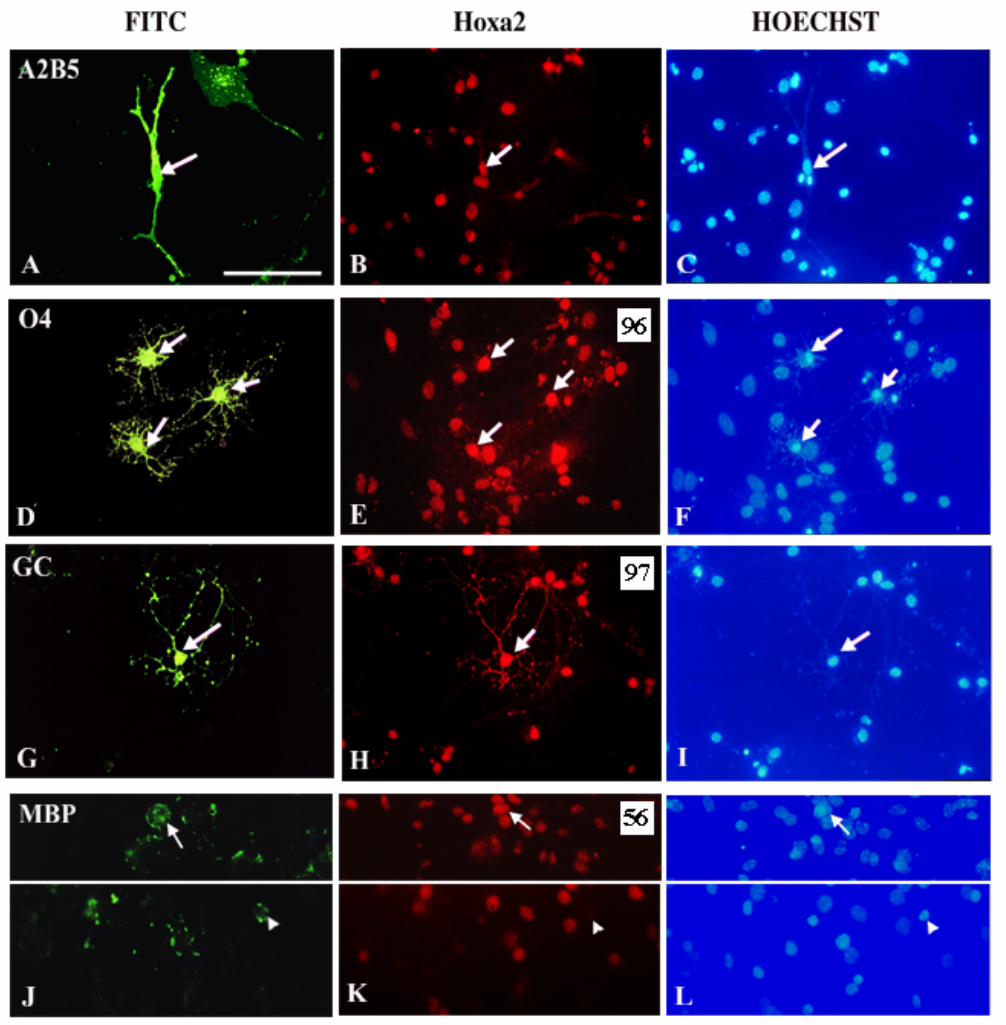


Figure 3.3 Hoxa2 is expressed throughout oligodendrocyte development.

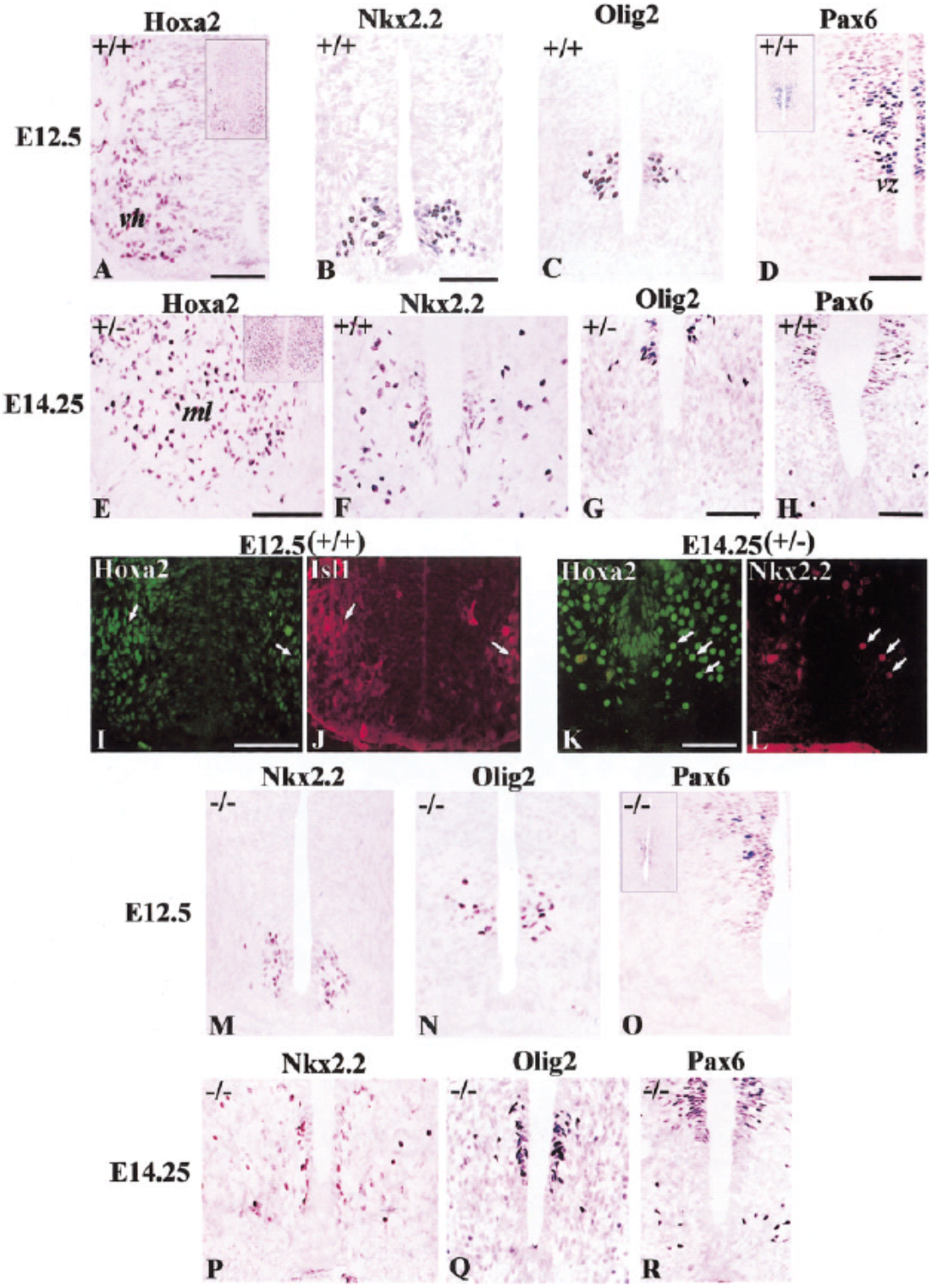
Primary mixed glial cell cultures from neonatal murine cerebral hemispheres were grown in culture for 18 days. Double immunofluorescent staining was conducted with each of the four OG markers [A2B5 (A), O4 (D), GalC (G), and MBP (J)] and Hoxa2 (B, E, H, K). Cells were visualized using nuclear fluorescent Hoechst counterstain (C, F, I, L). Each row illustrates micrographs obtained from individual filters [fluorescein (A, D, G, J), rhodamine (B, E, H, K), and DAPI (C, F, I, L)] of the same field. Arrows demarcate representative Hoxa2-immunoreactive cells. Arrowheads demarcate a $Hoxa2^{-}MBP^{+}$ cell. The insets at the top right corner of the rhodamine micrograph (E,H,K) show the percentage of $O4^{+}$, $GalC^{+}$, and MBP^{+} cells that are immunoreactive for Hoxa2, respectively. [Percentages depict averages calculated from two coverslips in which 100-200 OGs were counted. The percentage of $A2B5^{+}Hoxa2^{+}$ cells was not determined because the monoclonal A2B5 antibody binds to cell surface antigens on O-2A precursor cells, which can give rise to either OGs or type 2 astrocytes depending on culture conditions (Abney et al. 1983; Raff et al. 1983) and, therefore, is not specific to the OG lineage.] Scale bar = 100µm.

Figure 3.4 Transcription factor expression during oligodendrocyte specification in the spinal cords of *Hoxa2*+/+, *Hoxa2*+/-, and *Hoxa2*-/- mice.

(Top two rows) Transverse thoracic spinal cord sections from E12.5 (C16/T19) (A-D) and E14.25 (C19+/T21) (E-H) *Hoxa2*+/+ and *Hoxa2*+/- mice were immunolabeled with anti-Hoxa2 (A,E), anti-Nkx2.2 (B,F), anti-Olig2 (C,G) and anti-Pax6 (D,H) as described in Materials and Methods. Initially Hoxa2 expression was localized to the ventral horns (*vh*) at E12.5 (A), after which it was expressed throughout the mantle layer (*ml*) (E). Nkx2.2^{+} and Olig2^{+} cells, which were initially localized to distinct domains in the vVZ at E12.5 (B,C) had begun to migrate into the *ml* by E14.25 (F,G). Pax6 was expressed in the ventricular zone (*vz*) at both stages (D,H), with occasional immunoreactive cells found in the *ml* by E14.25 (H).

(Middle row) Transverse thoracic spinal cord sections from E12.5 (I,J) and E14.25 (K,L) *Hoxa2*+/+ and *Hoxa2* +/- mice were double immunolabeled with anti-Hoxa2 (I,K) and anti-Islet1(Isl1) (J) or anti-Nkx2.2 (L), respectively. Each fluorescent image illustrates micrographs obtained from individual filters [fluorescein (I,K) and rhodamine (J,L)] of the same field. Hoxa2 was co-expressed by Islet1 (Isl1)$^{+}$ MNs at E12.5 (I,J). At E14.25 Hoxa2^{+}Nkx2.2^{+} cells were demonstrated in the mantle layer (K,L).

(Bottom two rows) Transverse thoracic spinal cord sections from E12.5 (M-O) and E14.25 (P-R) *Hoxa2*-/- mice were immunolabeled with anti-Nkx2.2 (M,P), anti-Olig2 (N,Q), and anti-Pax6 (O,R). Expression profiles of Nkx2.2, Olig2, and Pax6 were similar in the presence (B-D, F-H) and absence (M-R) of *Hoxa2*. Analyses were conducted with spinal cord sections from at least three embryos at each stage of development in both the presence and absence of *Hoxa2*. Significant discrepancies were not observed and any inconsistencies were primarily due to the way the sections were cut, in particular, the location (C,Q) and shape (H,R) of the central canal varied between some sections. Scale bar = 100μm.


Hoxa2
Nkx2.2
Olig2
Pax6
E12.5
+/+
vh
vz
A
B
C
D
Hoxa2
Nkx2.2
Olig2
Pax6
E14.25
+/-
+/+
ml
E
F
G
H
E12.5(+/+)
E14.25(+/-)
Hoxa2
Isl1
Hoxa2
Nkx2.2
I
J
K
L
Nkx2.2
Olig2
Pax6
E12.5
-/-
M
N
O
Nkx2.2
Olig2
Pax6
E14.25
-/-
P
Q
R

profiles are altered in the absence of *Hoxa2*. *Hoxa2*+/+ and *Hoxa2*+/- mice were used interchangeably to represent the presence of *Hoxa2*, since expression profiles were similar in both genotypes. Single immunohistochemical analysis of *Hoxa2*+/+ mice demonstrated that at E12.5 (C16/T19) Olig2 is expressed in an adjacent non-overlapping domain with Nkx2.2, whereas Pax6 is expressed dorsal to the Nkx2.2$^+$ domain [Figure 3.4B–D]. Comparison of these expression profiles with those obtained from *Hoxa2*-/- mice demonstrated that they are maintained in the absence of *Hoxa2* [Figure 3.4M-O]. At E14.25 (C19+/T21), when immunoreactive cells are still evident in the vVZ, expression profiles of Nkx2.2, Olig2, and Pax6 are similar regardless of genotype [Figure 3.4F–H,P–R]. Double immunofluorescent staining of Nkx2.2 and Olig2 or Pax6 at E12.5 (C16/T19) confirmed that the expression profiles are maintained in the absence of *Hoxa2* [Figure 3.5].

3.4.3 *Hoxa2* is not required for early maturation of oligodendrocytes in the murine spinal cord

At E16 (C23/T23) and E18 (T25), Hoxa2 is expressed throughout the mantle layer, with occasional immunoreactive cells also observed in the marginal layer [Figure 3.6A,E,I–K]. Nkx2.2$^+$ and Olig2$^+$ cells continue to migrate into the mantle layer, and eventually these cells begin to accumulate in the marginal layer of *Hoxa2*+/+ and *Hoxa2*+/- mice. This migration is accompanied by a reduction and the eventual disappearance of Nkx2.2- and Olig2- immunoreactive cells in the vVZ at E16 (C23/T23) and E18 (T25), respectively [Figure 3.6B,C,F,G]. Comparisons of Nkx2.2 and Olig2 expression profiles from *Hoxa2*+/+ and *Hoxa2*+/- mice with that of *Hoxa2*-/- mice failed to reveal any consistent changes in expression profiles [Figure 3.6B–D,F–H,L–Q]. Double labeling immunohistochemical analysis of Olig2 and Nkx2.2 expression in *Hoxa2*+/+ and *Hoxa2*+/- mice demonstrated that with continued maturation, co-expression of these TFs becomes evident, especially in the marginal layer [Figure 3.7A,B]. Comparison of these co-expression profiles with those obtained from *Hoxa2*-/- mice show no detectable alterations [Figure 3.7C,D]. In addition, comparisons of *Hoxa2*+/+, *Hoxa2*+/-, and *Hoxa2*-/- mice demonstrated that the accumulation of Olig2$^+$ cells in the marginal layer of the developing spinal cord proceed similarly regardless of genotype [Figure 3.7E–H]. Thus, the early maturation of OGs proceeds

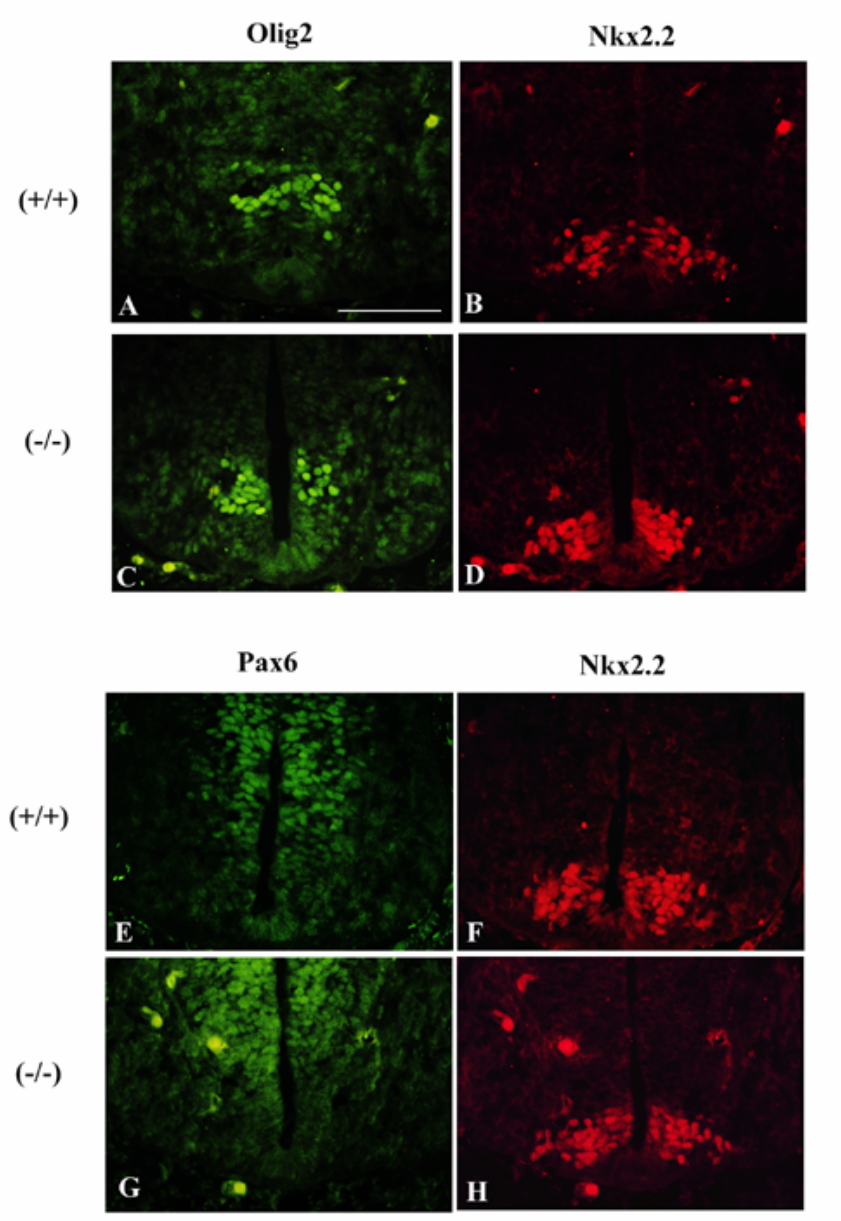


Figure 3.5 Oligodendrocyte specification occurs normally in the absence of *Hoxa2*.

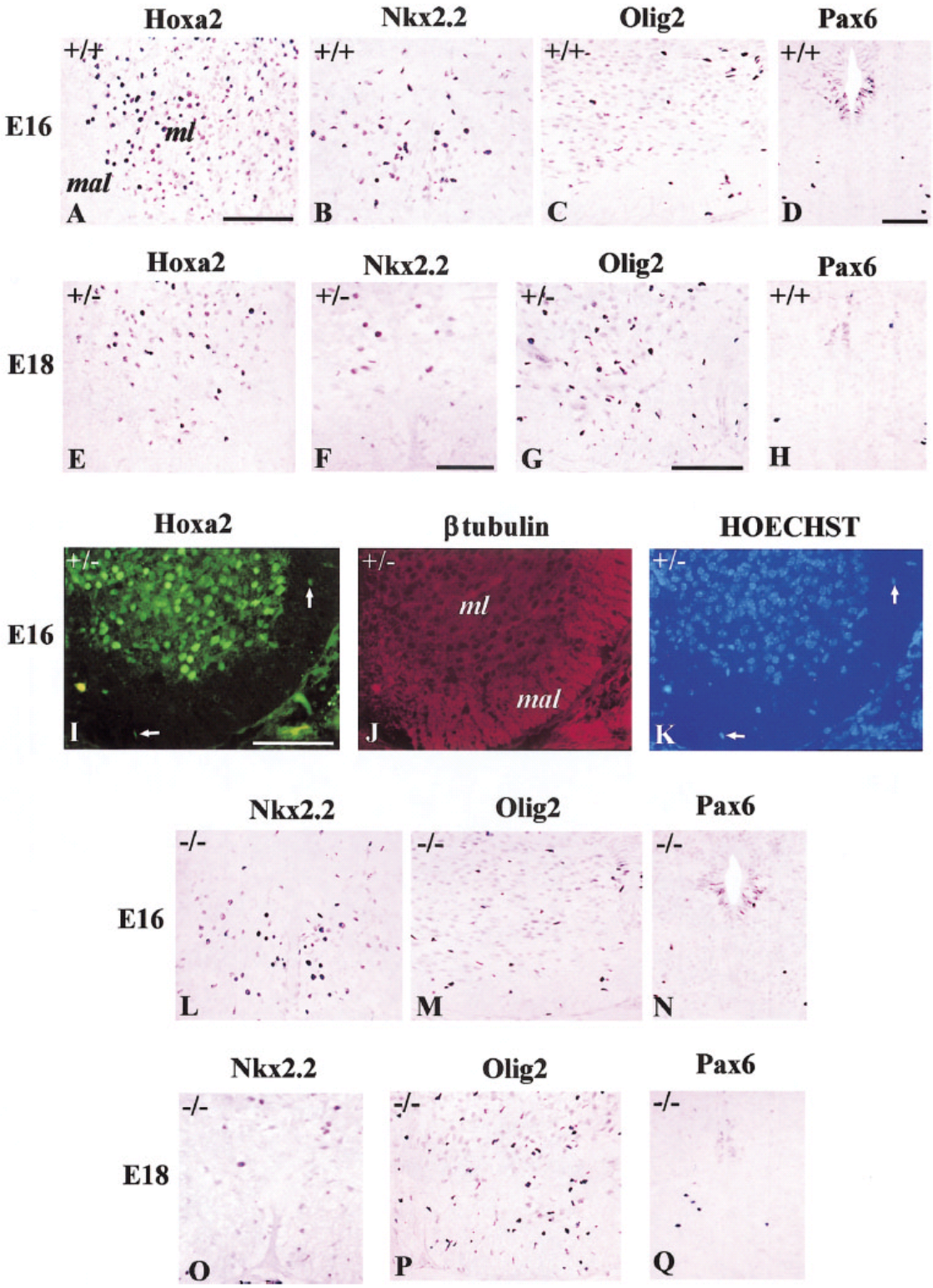
Transverse thoracic E12.5 (C16/T19) *Hoxa2*+/+ and *Hoxa2*-/- spinal cord sections were subjected to double immunofluorescent labeling with anti-Nkx2.2 and anti-Olig2 (A-D) or anti-Nkx2.2 and anti-Pax6 (E-H) antibodies. Each row illustrates micrographs obtained from individual filters [fluorescein (A, C, E, G) and rhodamine (B, D, F, H)] of the same field. Nkx2.2 and Olig2 exhibit adjacent non-overlapping expression domains in both WT (A,B) and mutant (C,D) mice. Nkx2.2 and Pax6 also exhibit similar expression domains in both WT (E,F) and mutant (G,H) mice. Scale bar = 100μm.

Figure 3.6 Transcription factor expression during oligodendrocyte maturation in the spinal cords of *Hoxa2+/+*, *Hoxa2+/-* and *Hoxa2-/-* mice.

(Top two rows) Transverse thoracic spinal cord sections from E16 (C23/T23) (A-D) and E18 (T25) (E-H) *Hoxa2+/+* and *Hoxa2+/-* mice were immunolabeled with anti-Hoxa2 (A,E), anti-Nkx2.2 (B,F), anti-Olig2 (C,G), and anti-Pax6 (D,H) as described in Materials and Methods. At these stages Hoxa2 was expressed predominantly in the *ml* (A,E). $Nkx2.2^{+}$ and $Olig2^{+}$ cells continued to migrate into the *ml* and eventually accumulated in the *mal* (B,C,F,G). Pax6 expression was concentrated in the ventricular zone at these stages, with occasional immunoreactive cells found in the *ml* (D,H).

(Middle row) Transverse thoracic spinal cord section from an E16 (I-K) *Hoxa2+/-* mouse was double immunolabeled with anti-Hoxa2 (I) and anti-β-tubulin (J). β-tubulin staining was used to demarcate the *mal*, which is populated primarily by glial cells. Cells were visualized using nuclear fluorescent Hoechst counterstain (K). Each fluorescent image illustrates micrographs obtained from individual filters [fluorescein (I), rhodamine (J), and DAPI (K)] of the same field. At later stages of development occasional $Hoxa2^{+}$ cells (arrows) were found in the glial-rich *mal*.

(Bottom two rows) Transverse thoracic spinal cord sections from E16 (L–N) and E18 (O–Q) *Hoxa2-/-* mice were immunolabeled with anti-Nkx2.2 (L,O), anti-Olig2 (M,P), and anti-Pax6 (N,Q). Expression profiles of Nkx2.2, Olig2, and Pax6 were similar in both the presence (B–D,F–H) and absence (L–Q) of *Hoxa2*. Scale bars=100 μm.


Hoxa2
Nkx2.2
Olig2
Pax6
E16
+/+
ml
mal
A
B
C
D
E18
+/-
E
F
G
H
βtubulin
HOECHST
I
J
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-/-
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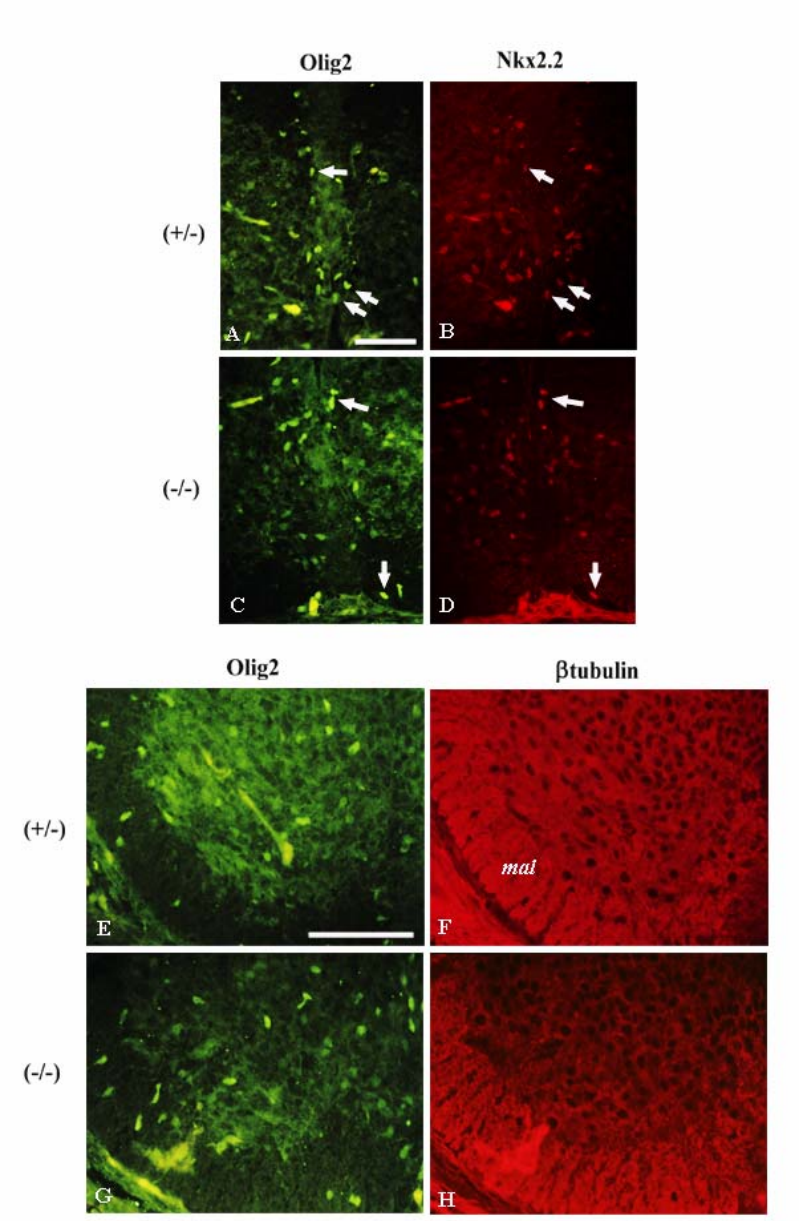


Figure 3.7 Early oligodendrocyte maturation proceeds normally in the absence of *Hoxa2*.

Transverse thoracic E16 (C23/T23) *Hoxa2+/-* and *Hoxa2-/-* spinal cord sections were subjected to double immunofluorescent labeling with anti-Nkx2.2 and anti-Olig2 (A-D) or anti-Olig2 and anti-β-tubulin (E-H) as described in Materials and Methods. Each row illustrates micrographs obtained from individual filters [fluorescein (A, C, E, G) and rhodamine (B, D, F, H)] of the same field. With continued maturation, cells began to co-express Olig2 and Nkx2.2 (arrows) in both heterozygous (A, B) and mutant (C, D) mice. Furthermore, the migratory pattern of $Olig2^{+}$ cells was similar in both the heterozygous (E, F) and mutant (G, H) mice, as demonstrated by the accumulation of immunoreactive cells in the marginal layer (*mal*). Scale bar = 100μm.

normally in the absence of the *Hoxa2* gene.

3.5 Discussion

OG development *in vitro* progresses through a number of distinct stages, which are characterized by specific antigenic phenotypes [Figure 3.2]. During the early stages of oligodendrogenesis, OPCs and pro-OGs, which are characterized by A2B5 and/or O4 staining, actively proliferate and migrate. Subsequently, loss of these traits and the emergence of GalC expression signal terminal differentiation into pre-myelinating OGs. Finally, progression to the mature myelinating phenotype occurs with the production of myelin proteins and the elaboration of sheet-like membranes (Abney et al. 1983; Duchala et al. 1995; Fok-Seang and Miller 1994; Miller 1996; Noble et al. 1988; Raff et al. 1985).

Although *in situ* hybridization and immunohistochemical analysis have shown that *Hoxa2* is expressed in regions of the CNS, including the myelencephalon, diencephalon, and spinal cord (Tan et al. 1992; Wolf et al. 2001), Hao et al. (1999) were the first to demonstrate the expression of Hoxa2 by cells of the OG lineage. In particular, Hao et al. (1999) demonstrated that $O4^+$ pro-OGs, obtained from E15 murine cerebral hemispheres, expressed Hoxa2. To confirm these findings, as well as further investigate Hoxa2 expression in cells of the OG lineage, an immunocytochemical analysis was performed on primary mixed glial cultures obtained from the cerebral hemispheres of newborn mice. These cultures consisted of oligodendroglia that developed on top of an astrocytic monolayer. The results showed that although Hoxa2 is expressed throughout OG development [Figure 3.3], the location of immunoreactivity, as well as the number of $Hoxa2^+$ OGs changes with maturation. In particular, Hoxa2 expression by OGs is primarily nuclear [Figure 3.3B,E], however, cytoplasmic staining is also observed at the pre-myelinating stage [Figure 3.3H]. The reason for this cytoplasmic staining is unknown, but it has been demonstrated with several other TFs (Armstrong et al. 1995; Wang et al. 2001). Subsequently, there is a reduction in the number of $Hoxa2^+$ myelinating OGs in comparison to $Hoxa2^+$ pre-myelinating OGs [Figure 3.3H,K].

Researchers recently identified a subfamily of bHLH TFs known as the *Olig* genes, whose members include Olig1, Olig2, and Olig3 (Lu et al. 2000; Takebayashi et al. 2000; Zhou et al. 2000). *Olig1* and *Olig2* are co-expressed by cells in the pMN domain of the developing spinal cord prior to *PDGFαR* (Lu et al. 2000; Zhou et al. 2000). In

contrast, Olig3, which is transiently expressed in the embryonic CNS, is not co-expressed with Olig2 (Takebayashi et al. 2002). *Olig1*$^+$ and *Olig2*$^+$ cells disperse throughout the spinal cord in a ventral to dorsal manner similar to that of *PDGFαR*$^+$ cells (Zhou et al. 2000). Furthermore, double *in situ* hybridization histochemical analysis has also demonstrated that *PDGFαR*$^+$ cells co-express *Olig1* and *Olig2* (Lu et al. 2000; Zhou et al. 2000). Hence, they are believed to be the earliest known markers of the OG lineage. In addition, *Olig1* and *Olig2* continue to be expressed in OGs into adulthood (Lu et al. 2000; Takebayashi et al. 2000)

In the present study, Olig2 was used to identify cells of the PDGFαR$^+$ OPC lineage. Although Olig2 initially specifies MN precursors at the early stages of spinal cord development, it is down-regulated as the cells differentiate (Novitch et al. 2001). In addition, its mRNA is not expressed by GFAP$^+$ astrocytes (Takebayashi et al. 2000), which are also found in the CNS. These findings suggest that during periods of oligodendrogenesis in the spinal cord, Olig2 is specific to the OG lineage. Olig2 was used as our OG marker instead of Olig1 for two reasons. First, whereas the number of *Olig2*$^+$ cells has been shown to be similar to that of *PDGFαR*$^+$ cells, *Olig1*$^+$ cells appear to out-number PDGFαR$^+$ cells (Zhou et al. 2000). Second, Olig2 has been shown to be critical for OG development in the spinal cord since OPCs and mature OGs fail to develop in its absence. In contrast, *PDGFαR*$^+$ cells appear on schedule in the absence of Olig1; however, there is a delay in the appearance of *Sox10*, *MBP*, and *PLP/DM20* expression in the white matter of the spinal cord (Lu et al. 2002).

Research on OG development in the chick has led to the hypothesis that a separate OPC lineage may also arise from the Nkx2.2$^+$ p3 domain. Although all migratory Olig2$^+$ cells co-express Nkx2.2 in the chick spinal cord, Nkx2.2$^+$ cells that are weak or negative for Olig2 expression are found, and a portion of these cells express O4 (Fu et al. 2002; Zhou et al. 2001). In addition, ~33% of A2B5$^+$ glial restricted precursor cells, which were immunopurified from ~E13.5 rat spinal cords, have been shown to be immunoreactive for Nkx2.2. These Nkx2.2$^+$ cells are believed to be OGs because few β-tubulin$^+$ neurons and GFAP$^+$ astrocytes express Nkx2.2 (Qi et al. 2001).

In our study, immunohistochemical analyses were initiated at E12.5 (C16/T19) since Olig2 expression would be down-regulated in postmitotic MNs at this time. In

addition, it corresponds to the emergence of *PDGFαR* expression in the vVZ (Sun et al. 1998). At this stage, Hoxa2 is expressed predominantly in the presumptive ventral horns [Figure 3.4A], which is in direct contrast to Nkx2.2 and Olig2, which are expressed in the vVZ [Figure 3.4B,C] of *Hoxa2*+/+ and *Hoxa2*+/- mice. Hence, our results suggest that Hoxa2 expression in OPCs occurs following specification. At later stages, overlapping expression profiles of Hoxa2 with Nkx2.2 and Olig2 in the mantle layer suggest that Hoxa2 is expressed by migratory OG cells. Unfortunately, we were unable to conclusively show double labeling of Hoxa2 and Olig2 because both antibodies are rabbit polyclonals. However, we were able to demonstrate co-expression of Hoxa2 by $Nkx2.2^+$ cells [Figure 3.4K,L], which have been hypothesized to represent a separate population of OPCs (Fu et al. 2002), as well as co-expression of Nkx2.2 and Olig2 [Figure 3.7A–D].

With continued maturation Hoxa2 expression in OGs appears to diminish as suggested by the finding that $Nkx2.2^+$ and $Olig2^+$ cells greatly out-number $Hoxa2^+$ cells in the marginal layer [Figure 3.6A–C,E–G]. In contrast, $Hoxa2^+$ cells out-number $Nkx2.2^+$ and $Olig2^+$ cells in the mantle layer, which supports the *in vitro* finding that cells in addition to OGs also express Hoxa2 [Figure 3.6A–C,E–G]. For example, Hoxa2 has been shown to be expressed by $Islet1^+$ MNs and $GFAP^+$ astrocytes (Hao et al. 1999) [Figure 3.4I,J]. Although Hoxa2 is not specific to the OG lineage, no TFs have been shown to be expressed exclusively by OGs.

As most research regarding OG development has involved the analysis of the rodent spinal cord, we decided to focus our study on this area of the CNS. Although Shh dependence and the ventral origin of OPCs are similar in the spinal cord and brain (Alberta et al. 2001; Hall et al. 1996; Ono et al. 1995; Orentas et al. 1999; Pringle and Richardson 1993; Tekki-Kessaris et al. 2001; Woodruff et al. 2001), differences in OG development in these regions have emerged. For example, $Nkx2.2^+/Olig2^+$ cells are found in the vVZ of the chick spinal cord prior to emigration (Fu et al. 2002; Zhou et al. 2001). In contrast, in the chick hindbrain only $Nkx2.2^+$ cells are found in the vVZ at the onset of oligodendrogenesis with *Olig2* expression only evident in migratory cells (Fu et al. 2002). Takebayashi et al. (2000) have also shown discrepancies in *Olig1* and *Olig2* expression in the murine embryonic brain versus spinal cord. In particular, in the murine embryonic brain *Olig2* displays a broader expression domain than *Olig1*. In addition, it

was shown that in certain brain regions numerous *Olig2*$^{+}$ cells are found, few of which co-express *Olig1* or *CNP* (Takebayashi et al. 2000), the latter being a myelinating glial cell specific enzyme. Takebayashi et al. (2000) concluded that *Olig2* may not be limited to OGs in the brain and, hence, may play a role in this region distinct from Olig1. This conclusion is supported by the study of Lu et al. (2002), who found that in the hindbrain and areas of the forebrain, the early stages of OG development proceed largely unaltered in the absence of *Olig2*. Hence, Olig2 does not appear to play a critical role in OG development in certain brain regions. These discrepancies may be partially due to differing expression profiles of regulatory TFs by cells of the OG lineage along the anterior-posterior axis, such as the *Hox* genes.

One potential role for Hoxa2 in OG development may be via its transcriptional regulation of NCAM. NCAM, a cell surface glycoprotein, exists as three prominent protein isoforms (120, 140, 180 kD), which are encoded by four predominant transcripts arising from alternative splicing and polyadenylation sites of a single gene (Barbas et al. 1988). Examination of the 5′ end of the *NCAM* gene has revealed two homeodomain binding sites (HBSs: I and II) that have the potential to be regulated by *Hox* genes (Hirsch et al. 1990; Jones et al. 1993; Jones et al. 1992). *Hoxc6* (*Hox3.3*) and *Hoxb9* (*Hox2.5*) have previously been shown to significantly enhance chloramphenicol acetyltransferase (CAT) activity via NCAM HBS (Jones et al. 1993; Jones et al. 1992). In contrast, *Hoxb8* (*Hox2.4*) decreases HBS-I^{+}/ HBS-II^{+}- directed CAT activity, as well as *Hoxb9*-enhanced activity (Jones et al. 1992). Hence, NCAM promoter activity may be the result of the combined actions of numerous *Hox* genes. Furthermore, mutations in NCAM HBSs results in alterations in β-galactosidase reporter gene expression patterns in the embryonic spinal cord *in vivo* (Wang et al. 1996).

NCAMs undergo a variety of post-translational modifications, including glycosylation by enzymes such as sialyltransferase (Breen et al. 1987; Breen and Regan 1988; Finne et al. 1983; Rothbard et al. 1982); the activity of this enzyme is developmentally regulated (Breen et al. 1987; Breen and Regan 1988). As a result, NCAMs vary in their sialic acid content, with the highest evident in PSA-NCAM (Breen et al. 1987; Breen and Regan 1988; Finne et al. 1983; Rothbard et al. 1982). Sialic acid content has been demonstrated to be inversely related to NCAM homophilic binding

properties, such as adhesion and rate of aggregation (Hoffman and Edelman 1983; Sadoul et al. 1983). Hence, Hoxa2 could potentially enhance or repress transcription of the *NCAM* gene, which would subsequently give rise to embryonic or adult forms, depending on the activity of sialyltransferase. The expression of either the embryonic or adult form of NCAM by oligodendroglial cells may affect their motility and maturation (Gard et al. 1996; Wang et al. 1994).

Comparison of Nkx2.2, Olig2, and Pax6 expression profiles obtained from spinal cord sections of at least three E12.5, E14.25, E16, and E18 mice in both the presence (*Hoxa2*+/+ and *Hoxa2*+/-), and the absence of *Hoxa2* (*Hoxa2*-/-) failed to reveal major differences. These results suggest that Hoxa2 does not appear to be critical for the OG specification and early maturation in the murine spinal cord. One potential reason for this could be that other *Hox* genes may compensate for the loss of *Hoxa2* in the developing spinal cord. Analyzing OG development in the more anterior regions of the CNS in *Hoxa2*-/- mice, where *Hox* gene compensation is less likely, should demonstrate whether discrepancies in early stages of OG development exist in the absence of the *Hoxa2* gene.

The regulatory mechanisms controlling oligodendrogenesis, once elucidated, will guide the development of therapeutic approaches to treating demyelinating diseases such as MS. MS is an inflammatory disease of the CNS characterized by focal areas of demyelination that appear to be immune driven (Lucchinetti et al. 1999; Ozawa et al. 1994; Prineas et al. 1993a; Prineas et al. 1993b; Raine 1997a; Raine 1997b; Storch et al. 1998). The OG progenitors are the most motile of the OG lineage cells (Gansmuller et al. 1991) that are recruited to areas of demyelination over only a very limited area (Franklin and Blakemore 1997). There are three potential sources of remyelinating OGs: adult progenitors endogenous to or adjacent to the lesion, or mature OGs that have survived the demyelinating event (Levine et al. 2001). Remyelination, which is characterized by axons exhibiting thin myelin sheaths, fails with disease progression (Brück et al. 1994; Ozawa et al. 1994; Prineas et al. 1993a; Prineas et al. 1993b; Prineas et al. 1989; Raine and Wu 1993). The reason that remyelination fails remains unknown, but it may include errors in migration or maturation. PSA-NCAM is one of several molecules that appear to be important for the migratory ability of OG progenitor cells (Barral-Moran et al. 2003).

Hence, elucidation of the transcriptional control by *Hox* genes of molecules involved in these processes, such as NCAMs, will help determine ways to enhance remyelination.

Preamble to Chapter 4

The purpose of this chapter is to determine if other Hox TFs could potentially compensate for Hoxa2 in the spinal cord in its absence. Given the fact that *Hoxa2* is one of 39 murine *Hox* genes, which exhibit extensive overlapping expression profiles in the spinal cord, I decided to examine the expression of an additional Hox TF, Hoxb4, during OG development.

(Manuscript published, Appendix C)

Nicolay DJ, Doucette JR, Nazarali AJ. 2004b. Hoxb4 in oligodendrogenesis. Cell Mol Neurobiol 24:357-366.

4.0 Hoxb4 IN OLIGODENDROGENESIS (Manuscript published)

4.1 Summary

1. Although recent advances have provided insight into the transcriptional control of OG development, little information exists on the role of clustered *Hox* genes in this process. The aim of this study is to examine the expression profile of Hoxb4 in the oligodendroglial lineage.
2. Immunocytochemical analysis of primary mixed glial cultures demonstrated that Hoxb4 is expressed throughout OG development, being co-expressed with oligodendroglial markers, A2B5, O4 (97%), GalC (91%), and MBP (93%).
3. Immunohistochemical analysis of transverse spinal cord sections demonstrated diffuse expression of Hoxb4 throughout the spinal cord at E12.5 (C16/T19), after which expression is confined primarily to the presumptive gray matter.
4. At E14.25 (C19C/T21), $Olig2^+$ cells begin to migrate out from the vVZ into the presumptive gray matter. These results suggest that $Olig2^+$ cells may co-express Hoxb4 since it is expressed throughout this region.
5. The expression of Hoxb4 by cells of the OG lineage indicates that it could play a role in OG maturation.

4.2 Introduction

OGs are myelinating cells of the CNS. In the murine spinal cord $PDGF\alpha R^+$ OPCs first appear at approximately E12.5 in a distinct region of the vVZ (Sun et al. 1998). This region has been shown to correspond to the pMN domain, which is defined by the expression of the bHLH gene *Olig2* (Novitch et al. 2001; Zhou et al. 2000). Although Olig2 initially specifies MN precursors at early stages of spinal cord development, it is down-regulated as these precursor cells differentiate into MNs (Mizuguchi et al. 2001; Novitch et al. 2001; Zhou et al. 2000). *Olig2* expression that persists in the pMN domain after MNs are generated specifies cells of the OG lineage (Lu et al. 2000; Zhou et al. 2000). Initially only a few OPCs are found on each side of the central canal but these cells subsequently proliferate and migrate first laterally then dorsally to populate the entire cross-sectional area of the spinal cord (Lu et al. 2000; Zhou et al. 2000).

Analysis of glial cell differentiation *in vitro* has shown that OG development

progresses through a number of distinct stages, which are characterized by the expression of specific cell surface markers [Figure 4.1]. During early stages of differentiation, characterized by A2B5 and/or O4 staining, cells are actively proliferating and migratory. Loss of these traits signals terminal differentiation of the OG lineage, which is characterized by the expression of GalC and, subsequently, the expression of myelin proteins (Abney et al. 1983; Asou et al. 1995; Bansal et al. 1989; Duchala et al. 1995; Fok-Seang and Miller 1994; Miller 1996; Noble et al. 1988; Raff et al. 1985; Raff et al. 1983; Sommer and Schachner 1981).

The timing of OG differentiation is believed to be dependent on an intrinsic clock that counts cell divisions driven by mitogens, such as platelet-derived growth factor (PDGF) (Raff et al. 1985; Raff et al. 1988). Differentiation is further modified by hydrophobic signals, such as RA or thyroid hormone (Barres et al. 1994; Ibarrola et al. 1996; Noll and Miller 1994). Potential downstream targets of RA signaling are the *Hox* genes, which are characterized by a 180 base pair homeobox (Gould et al. 1998; McGinnis et al. 1984a; McGinnis et al. 1984b). This nucleotide sequence encodes a 60 amino acid homeodomain that contains a helix-turn-helix motif (Kissinger et al. 1990; McGinnis et al. 1984a; McGinnis et al. 1984b). Hence, Hox TFs can regulate the expression of downstream effector genes by binding to specific nucleotide sequences through the homeodomain (Han et al. 1989; Hoey and Levine 1988; Kissinger et al. 1990). *Hoxb4* is one of 39 mouse and human *Hox/HOX* genes that are organized into four clusters (*Hox* A, B, C, D) located on different chromosomes (Favier and Dollé 1997; Graham et al. 1989; Scott 1992). *In situ* hybridization histochemistry conducted by Gaunt et al. (1989) and Graham et al. (1988, 1989) demonstrated that *Hoxb4* mRNA is expressed in numerous tissues, including the CNS with the anterior limit of expression extending into the myelencephalon. We have analyzed Hoxb4 expression patterns during murine OG development in cell cultures and in spinal cord sections. Our results demonstrate that Hoxb4 is expressed by cells of the OG lineage.

4.3 Materials and Methods

4.3.1 Primary glial cultures

Cerebral hemispheres from newborn mice were dissected in DMEM/F12/10% FBS. Single cell suspensions, obtained by forcing diced tissue through a 75 μm Nitex

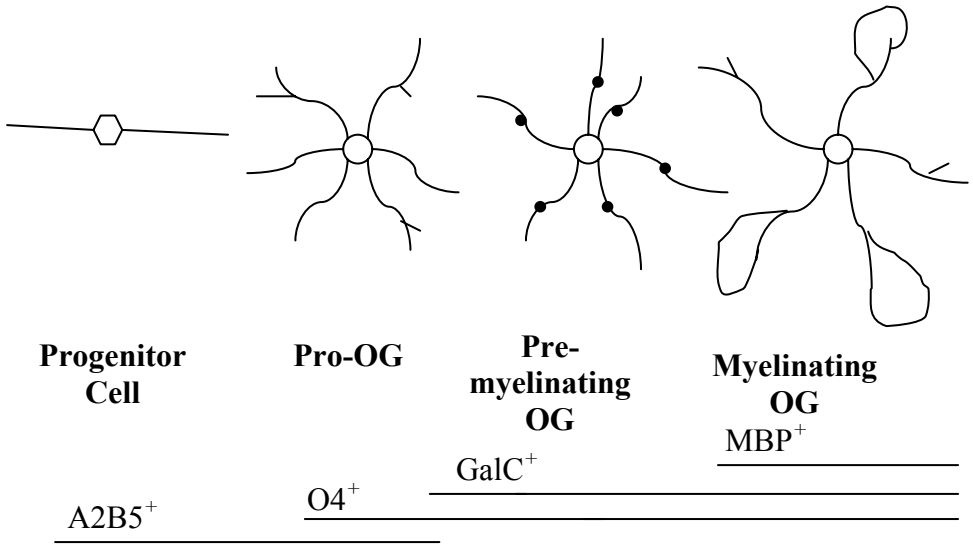


Figure 4.1 Illustration depicting oligodendrocyte development. Oligodendrocytes progress through four distinct stages which are characterized by unique morphological and antigenic phenotypes

(Abney et al. 1983; Asou et al. 1995; Bansal et al. 1989; Duchala et al. 1995; Fok-Seang and Miller 1994; Miller 1996; Noble et al. 1988; Raff et al. 1985; Raff et al. 1983; Sommer and Schachner 1981)

mesh, were plated in DMEM/F12/10% FBS for 4 days to allow adherence of the cells. They were then cultured for two 1-week periods, respectively, in a B104 conditioned medium and a low serum-containing (0.3% FBS) growth medium (GP medium) used previously by Gard and Pfeiffer (1990) and by Doucette and Devon (1994); this OG growth medium was supplemented with 10 ng/ml basic FGF. The growth medium was changed every 4 days.

4.3.2 Immunocytochemistry

The following primary antibodies were utilized for immunocytochemistry: anti-Hoxb4 (BabCO, CA; 1:500), A2B5 (ATCC; 1:10), O4 (hybridoma; Sommer and Schachner, 1981; 1:10), anti-GalC (BRD1 hybridoma; Ranscht et al. 1982; 1:20), and anti-MBP (Sternberger Monoclonals; 1:800). The cell cultures were double immunostained with Hoxb4 and A2B5, O4, or GalC using the live staining method described by Doucette and Devon (1994) and Hao et al. (1999) but with an incubation period of 30 min at 37°C for A2B5, O4, and anti-GalC. The secondary antibodies used included a goat anti-mouse IgM FITC (Sigma, ON; 1:100); a donkey anti-mouse IgG FITC (BIO/CAN Scientific, ON; 1:50); and a goat anti-rabbit IgG CY3 (BIO/CAN Scientific, ON; 1:200). Between incubations cultures were washed two times for 5 min each in PBS. Double immunostaining for Hoxb4 and MBP was done using the procedure described in Doucette and Devon (1994) with two modifications: (1) permeabilization and blocking were combined with a 30 min incubation in 3% SM/0.1% TX; (2) the primary antibodies were diluted in 1% SM/0.03% TX. The nuclei in all cell cultures were stained using HOECHST dye.

4.3.3 Immunohistochemistry

C57 black mouse embryos were staged according to the Carnegie (Butler and Juurlink 1987) and Theiler (1972) staging systems. The E and corresponding C/T stages utilized were E12.5 (C16/T19), E14.25 (C19+/T21), E16 (C23/T23), and E18 (T25); the Carnegie staging system does not go higher than stage 23. Once the embryos were staged, they were fixed by immersion in 4% formaldehyde at 4°C for 24–48 h depending on their age, and cryostat sections (8 μm) were cut through the upper thoracic level as described by Hao et al. (1999). The following primary antibodies were used: polyclonal anti-Olig2 (E12.5, 1:4000; rest of stages, 1:2000, gift of Dr Hirohide Takebayashi) and anti-Hoxb4

(E12.5, 1:3000; rest of the stages, 1:2000). Immunohistochemical staining was performed using the procedure described by Hao et al. (1999), with the following modifications: PBS washes were 8 min instead of 10 min; 3% SM/0.1% TX block/permeabilizations were 20 min versus 30–60 min; and incubation in the secondary antibody was 30 min versus 45 min.

4.4 Results and Discussion

Immunocytochemistry was conducted in this study as it allowed for a thorough investigation of Hoxb4 expression throughout OG development. The cerebral hemisphere cultures analyzed consisted of oligodendroglia that developed on top of an astrocytic monolayer. Although the anterior limit of *Hoxb4* expression has been shown to be the mid-myelencephalon (Gaunt et al. 1989; Graham et al. 1989; Graham et al. 1988), Hoxb4 is expressed by cells obtained from murine cerebral hemispheres. Similar findings have been observed with Hoxa2 where its expression was subsequently shown to extend to the forebrain (Hao et al. 1999; Tan et al. 1992; Wolf et al. 2001). Furthermore our laboratory has previously demonstrated that Hoxa2 is expressed by $O4^+$ pro-OGs obtained from E15 mouse cerebral hemispheres (Hao et al. 1999).

Hoxb4 is expressed by the majority of cells throughout OG development. Greater than 90% of the cells that express O4 (97%), GalC (91%), and MBP (93%) also co-express Hoxb4 [Figure 4.2]. The percentage of $A2B5^+/Hoxb4^+$ cells was not determined because A2B5 labels O-2A precursor cells, which can give rise to either OGs or type 2 astrocytes depending on culture conditions (Raff et al. 1983) and, therefore is not specific to the OG lineage. Expression of Hoxb4, which is a TF, is primarily nuclear [Figure 4.2B, E, K], however, weak cytoplasmic staining is observed occasionally in $GalC^+$ cells [Figure 4.2H]. The reason for this is unknown but similar observations have been reported with several other TFs (Armstrong et al. 1995; Wang et al. 2001).

Although Hoxb4 is expressed throughout OG development, it is not specific to the OG lineage. This is demonstrated by the fact that the number of $Hoxb4^+$ cells is greater than that of OG markers in primary mixed glial cultures [Figure 4.2]. Furthermore, *Hoxb4* RNA has been shown to be expressed in various embryonic tissues, including hindbrain, spinal cord, stomach, lung, kidneys, and prevertebra (Gaunt et al.1989; Graham et al. 1988).

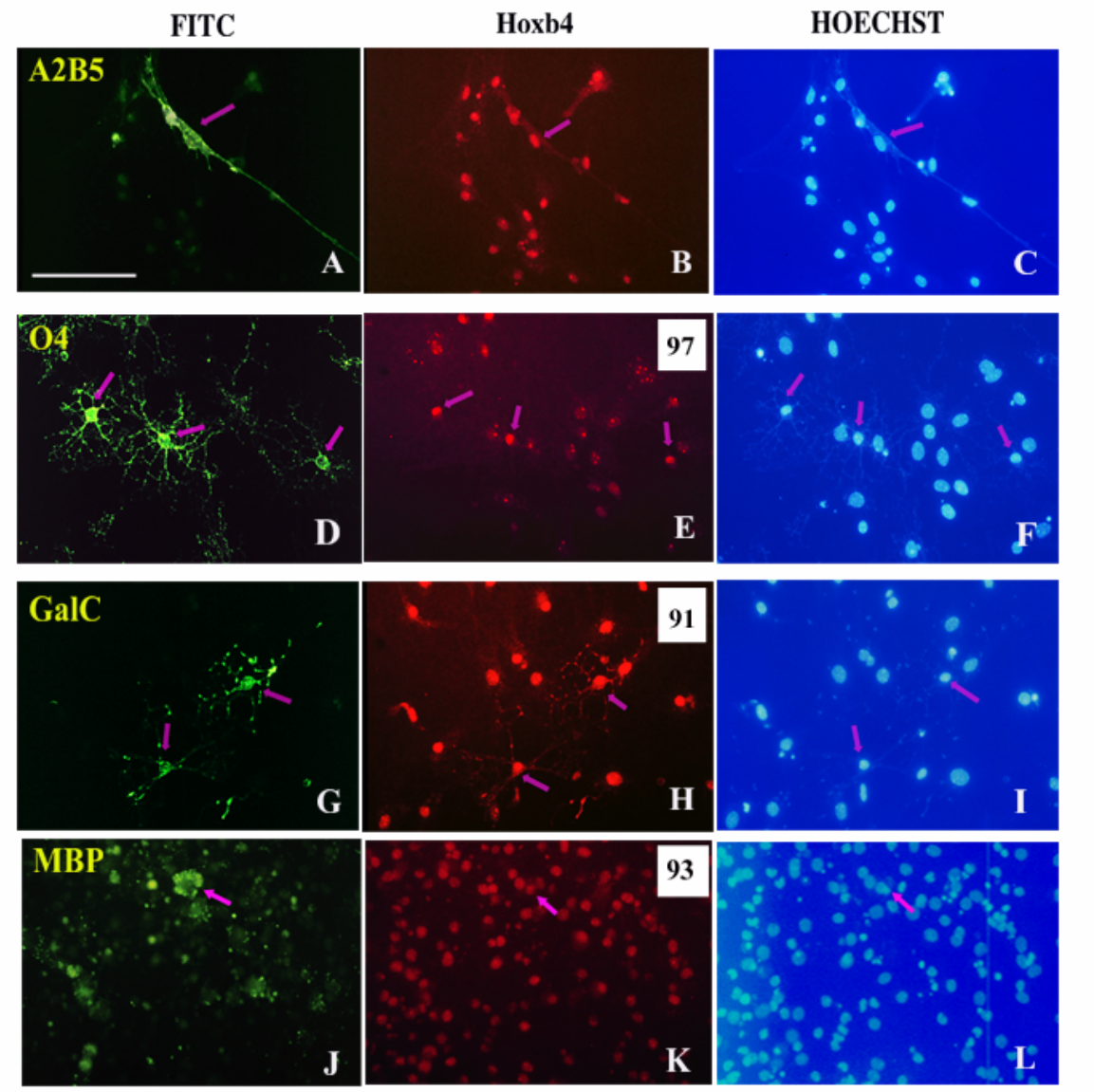


Figure 4.2 Hoxb4 expression pattern throughout oligodendrocyte development.

Primary glial cell cultures from neonatal murine brain were grown in culture for 18 days. Double immunofluorescent staining was conducted with each of the four OG markers [A2B5 (A), O4 (D), GalC (G), and MBP (J)] and Hoxb4 (B, E,H, K). Cells were visualized utilizing nuclear fluorescent HOESCHT counterstain. Each row illustrates micrographs obtained from individual filters [fluorescein (A, D, G, J), rhodamine (B, E, H, K), and DAPI (C, F, I, L)] of the same field. Arrows demarcate representative Hoxb4 immunoreactive cells. The insets at the top right corner of the rhodamine micrographs (E, H, L) show the percentage of $O4^+$, $GalC^+$, and MBP^+ cells that are immunoreactive for Hoxb4, respectively. [Percentages depict averages calculated from 2 to 3 coverslips in which 100–150 OGs were counted.] Bar = 100 μm for Fig. (A)–(L).

Hoxb4 expression was investigated in transverse spinal cord sections at stages E12.5 (C16/T19) to E18 (T25). At E12.5 (C16/T19) expression is diffuse being slightly more intense in the presumptive ventral horns [Figure 4.3A]. At later stages of development (E14.25–E18) Hoxb4 is also found in the presumptive dorsal horns [Figure 4.3C, E, G]. Previously, Graham et al. (1988, 1991) and Gaunt et al. (1989), had determined by *in situ* hybridization histochemistry the expression patterns of *Hoxb4*. Although only one micrograph of a 12.5 dpc transverse spinal cord section illustrated *Hoxb4* expression, it has been stated to exhibit a dorsal-ventral expression pattern similar to that of the other *Hoxb* genes (Graham et al. 1991; Graham et al. 1988). At 10.5 dpc, the expression of *Hoxb* genes is dispersed throughout the entire spinal cord (Graham et al. 1991; Graham et al. 1988). In our immunohistochemical analysis, immunoreactivity appears to be slightly greater in the presumptive ventral horns [Figure 4.3A]. *In situ* hybridization histochemistry has also shown that *Hoxb* genes exhibit strong expression in the dorsal spinal cord at 12.5 dpc after which expression appears in the ventral spinal cord at 14.5 dpc (Graham et al. 1991). In our study, Hoxb4 is found to be expressed throughout the presumptive gray matter at E14.25 (C19+/T21) [Figure 4.3C]. The *Hoxb4* gene has been shown to give rise to multiple transcripts (Graham et al. 1988); it is not known if all yield a translated product. The probe utilized by Graham et al. (1991) did not distinguish between them; therefore, differences in expression patterns may exist for the individual transcripts.

There are currently very few immunohistochemical markers available for studying OG development *in vivo*. Therefore, OG development in the spinal cord was followed by analyzing Hoxb4 expression in relation to that of Olig2. Evidence that supports the use of Olig2 as an OG marker is threefold. First, *Olig2*$^{+}$ cells, which are initially found in pMN domain, disperse throughout the spinal cord as previously described for *PDGFαR* (Lu et al. 2000; Zhou et al. 2000). Furthermore, it has been shown that the number of *Olig2*$^{+}$ cells is similar to that of *PDGFαR*$^{+}$ cells (Zhou et al. 2000). Second, *Olig2* is not co-expressed with the astrocytic marker GFAP (Takebayashi et al. 2000). Finally, Olig2 appears to be critical for OG development since in its absence OPCs and differentiated OGs fail to develop in the spinal cord (Lu et al. 2002). Therefore, *Olig2* is the earliest known marker of OG development since its expression precedes that of *PDGFαR* by

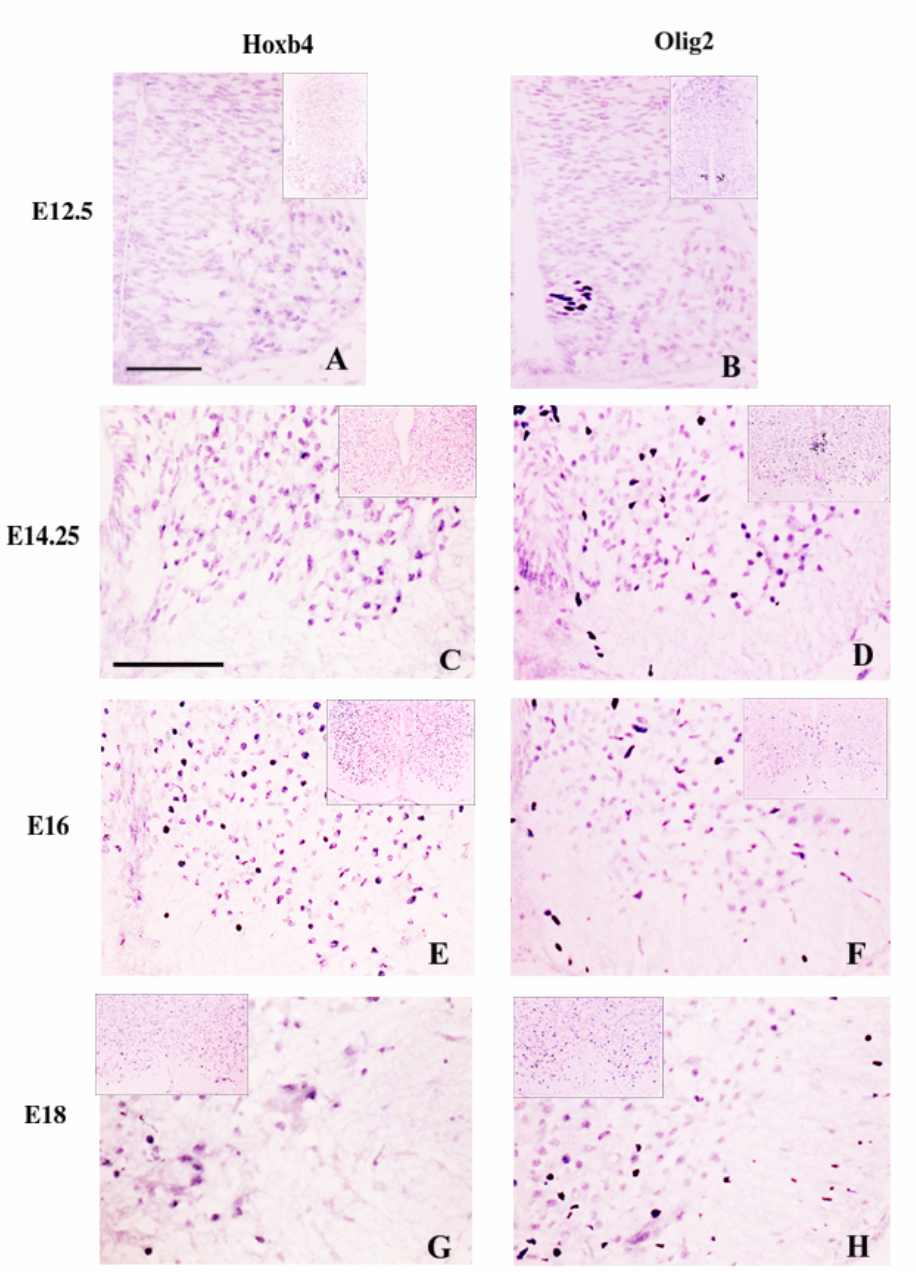


Figure 4.3 Expression of Hoxb4 in the embryonic spinal cord in relation to the oligodendrocyte marker Olig2.

Transverse sections of the thoracic spinal cord at E12.5 (C16/T19), E14.25 (C19C/T21), E16 (C23/T23), and E18 (T25) were immunolabeled with Hoxb4 (A, C, E, G) and Olig2 (B, D, F, H). At E12.5 when Olig2^{+} OPCs (B) were localized to the vVZ Hoxb4 (A) was diffusely expressed throughout the spinal cord, being slightly more intense in the ventral horns. Also at later stages of embryonic development Hoxb4's expression pattern (C, E, G) displayed domains of overlap with Olig2 (D, F, H). The micrographs are representative of sections obtained from approximately four embryos from at least two different litters. Bar = 100 μm for Fig. (A)–(H).

approximately 1–1.5 days (Zhou et al. 2000). Immunohistochemical analysis in this report has focused primarily on the ventral spinal cord due to the predominant expression of Olig2 in this region during embryonic development. We were unable to demonstrate co-expression of Hoxb4 with Olig2 since both antibodies are rabbit polyclonals.

Immunohistochemical analyses were initiated at E12.5 (C16/T19) since at this stage Olig2 expression has been down-regulated in differentiated MNs and *PDGFαR*$^+$ OPCs have arisen in the vVZ. Unlike Olig2, which is localized to the vVZ [Figure 4.3B], Hoxb4 stained nuclei are diffusely distributed at this stage, with immunoreactivity slightly more intense in the presumptive ventral horns [Figure 4.3A]. The Hoxb4$^+$ cells in the ventral horns are most likely neurons because neuron-specific β-tubulin is also expressed in these areas [Appendix B]. However, the results suggest that Hoxb4 may be expressed by OPCs *in vivo* since Hoxb4 stained nuclei are diffusely distributed throughout the ventricular zone. From E14.25 (C19+/T21) onward, Hoxb4 is expressed throughout the presumptive gray matter [Figure 4.3C,E,G]. Beginning at E16 (C23/T23), some Hoxb4$^+$ cells are also observed in the presumptive white matter, increasing somewhat in numbers at E18 (T25) [Figure 4.3E,G]. Olig2$^+$ cells begin to accumulate in the presumptive gray matter at ~E14.25 (C19+/T21) with occasional immunoreactive cells found in the presumptive white matter [Figure 4.3D]. This migratory pattern continues at later stages with an increasing number of immunoreactive cells accumulating in the presumptive white matter [Figure 4.3F,H]. Therefore, these results suggest that a population of Olig2$^+$ cells may co-express Hoxb4. However, the Olig2$^+$ cells out-number Hoxb4$^+$ cells in the presumptive white matter [Figure 4.3G,H]. In addition, the number of Hoxb4$^+$ cells in transverse spinal cord sections is greater than that of the Olig2$^+$ cells, which lends support to our cell culture findings that cells other than OGs express this Hox protein.

One potential role of Hoxb4 in OG development may be as a downstream effector in the RA pathway. The role of RA in OG development has previously been described. In particular, Noll and Miller (1994), utilizing E14–18 rat spinal cord cultures, found that RA (1 μM) inhibits maturation of OPCs. Interestingly, Barres et al. (1994) showed that low concentrations of RA (10 nM) applied to P8 rat optic nerve cultures promotes differentiation. Although these results initially appear to be contradictory it could be

hypothesized that they represent ends of the same developmental pattern. Hence, high concentrations of RA early on in development could inhibit OG differentiation, allowing OPC distribution throughout the spinal cord (Noll and Miller 1994). Then as OG development proceeds and RA levels diminish, cells could be facilitated to differentiate. Since RA has been shown to regulate *Hoxb4* expression in hindbrain (Gould et al. 1998), it has the potential to affect expression in OGs. Now that it has been demonstrated that Hoxb4 is expressed by cells of the OG lineage it will be interesting to determine whether RA exerts its effects on OG development via *Hox* genes, such as *Hoxb4*.

Preamble to Chapter 5

As the anterior boundary of most *Hox* genes is found in the hindbrain or spinal cord, it was decided to look at a more anterior region which would be less likely to have compensatory mechanisms at play. Hoxa2 is expressed in the telencephalon, which exhibits several similarities to the spinal cord in regards to the transcriptional control of oligodendrogenesis. Therefore, I investigated Hoxa2 expression, as well as its potential role(s) in OG development in this CNS region.

5.0 ROLE OF Hoxa2 IN TELENCEPHALIC MYELINATING GLIAL CELLS

5.1 Summary

Oligodendroglial cells express a dynamic combination of TFs as they progress through the various stages of development. Although the TF Hoxa2 is known to be expressed throughout OG development *in vitro* [Section 3.4.1, pg. 51], its role in this glial cell is currently unknown. Therefore, the aim of this study was to examine Hoxa2 expression and function in oligodendroglial cells in the telencephalon. Hoxa2 is expressed by oligodendroglial cells in this region; however, it does not appear to be required for OG specification or early maturation in the telencephalon. Immunocytochemical analysis was conducted on WT and *Hoxa2*-over-expressing CG4 cells to determine Hoxa2's effect on oligodendroglia differentiation. The results show that after four days CG4 cultures over-expressing *Hoxa2* contain significantly fewer $O4^+$ and $GalC^+$ cells than WT CG4 cultures. In order to determine the mechanism by which Hoxa2 could affect CG4 cell differentiation, we examined the expression of PSA-NCAM, which may impair this process. Contrary to our expectation, however, the results show that Hoxa2-Sense (Hoxa2-S) CG4 cultures contain significantly fewer PSA-$NCAM^+$ cells compared to WT. This finding suggests that Hoxa2's effect on CG4 cell differentiation is independent of PSA-NCAM. However, as PSA-NCAM is known to affect other aspects of OG development, such as migration, this result suggests that Hoxa2 could also play other roles in oligodendroglia development.

5.2 Introduction

In the developing telencephalon, OPCs arise in three distinct waves: first from the MGE/AEP, and then, subsequently, from the LGE/CGE, and, finally, from the dorsal telencephalon (Kessaris et al. 2006). OPCs must proliferate and migrate out from these domains in order to populate specific telencephalic regions (Kessaris et al. 2006) [Table 2.1]. In addition, they must progress through three subsequent stages of development—the pro-OG, pre-myelinating OG, and mature myelinating OG stages [Figure 2.4]— in order to myelinate axons in this region. As pre-myelinating OGs do not actively proliferate (Fok-Seang and Miller 1994) or migrate (Noble et al. 1988), the

proper progression of oligodendroglial cells through the various stages of development is critical to ensure normal myelination and, hence, CNS function.

Oligodendroglial cells express a dynamic combination of TFs as they progress through the various stages of oligodendrogenesis [Figure 2.4]. However, the function(s) of most of these TFs remains to be elucidated. One such TF is Hoxa2, which is one of 39 mouse and human *Hox/HOX* genes that are organized into four clusters (*Hox* A, B, C, D) located on different chromosomes (Akin and Nazarali 2005; Favier and Dollé 1997; Santagati and Rijli 2003; Scott 1992). Our initial attempts to elucidate Hoxa2's role in oligodendrogenesis in the embryonic spinal cord failed to reveal any noticeable differences in OG specification [Section 3.4.2, pg.52] or early maturation [Section 3.4.3, pg. 56] in *Hoxa2* transgenic knockout mice (Nicolay et al. 2004a). However, as *Hoxa2* is one of 39 murine *Hox* genes, which exhibit extensive overlapping expression profiles in the spinal cord (Akin and Nazarali 2005), other *Hox* genes could compensate for *Hoxa2* in this CNS region in its absence. As a result, in the present study, we have investigated Hoxa2's expression and function during oligodendrogenesis in the telencephalon. This region was chosen based on the fact that *Hoxa2* mRNA and protein is expressed in the telencephalon (Tan et al. 1992; Wolf et al. 2001), as well as by oligodendroglial cells found in primary mixed glial cultures obtained from this region (Hao et al. 1999; Nicolay et al. 2004a) [Section 3.4.1, pg. 51]. Furthermore, as the expression limit of most *Hox* genes is in the spinal cord or hindbrain (Akin and Nazarali 2005), the likelihood of compensatory effects by other *Hox* genes should be minimal in this CNS region. In turn, the telencephalon exhibits several similarities with the spinal cord in regards to the transcriptional control of oligodendrogenesis. The results of this study confirm that Hoxa2 is expressed by early oligodendroglial cells in the embryonic telencephalon. In addition, they suggest that Hoxa2 may affect multiple aspects of OG development, including differentiation.

5.3 Materials and Methods

5.3.1 *Hoxa2* transgenic and CD1 mice

Hoxa2+/+, *Hoxa2*+/-, and *Hoxa2*-/- C57 black mice (Gendron-Maguire et al., 1993) were acquired by timed heterozygous matings. Swiss CD1 and C57 black mice were staged according to the Carnegie (Butler and Juurlink, 1987) and Theiler (1972)

staging systems. The E and corresponding C/T stages used were as follows: E13.5 (C18/T20), E14.25 (C19+/T21) and E18 (T25); the Carnegie staging system does not go beyond stage 23. Although *Hoxa2*-/- embryos can be identified by external ear deformities at E14.25 (C19+/T21) onward, genotypes were confirmed by PCR analysis (Gendron-Maguire et al., 1993). The E13.5 (C18/T20) and E14.25 (C19+/T21) mouse embryos were fixed by immersion in 4% formaldehyde, which was made from paraformaldehyde, at 4°C. The E18 (T25) mouse embryos were perfused with 4% formaldehyde and subsequently post-fixed for 1 day. All embryos were then rinsed and stored in 20% sucrose until they were sectioned. Cryostat sections (8μm) were collected on gelatin-coated coverslips and allowed to dry for at least 1 h prior to staining. In the case of the E18 (T25) mice, the sections were stored at -20°C until they were processed via immunohistochemistry.

5.3.2 *In situ* hybridization histochemistry and immunohistochemistry

For *in situ* hybridization histochemistry (conducted by a former PhD student Louise Wolf), Swiss CD1 mice were fixed with 4% formaldehyde, embedded in OCT, and sectioned utilizing a cryostat. The sections (8 μm) were subsequently hybridized with *Hoxa2* and *Hoxd1* probes as described in Hao et al. (1999) and Wolf et al. (2001).

For immunohistochemical analysis the following primary antibodies were used: anti-Olig2 (a kind gift from Dr. Hirohide Takebayashi (Takebayashi et al., 2000); 1:6000), anti-Hoxa2 (Hao et al., 1999; 1:1000), anti-Pax6 (Developmental Studies Hybridoma Bank; 1:400) and anti-PDGFαR (BD Pharminigen™; 1:100). The protocol utilized for double labeling sections for Olig2 and Pax6 was previously described in Nicolay et al. (2004a) [Section 3.3.2, pg. 50]. For immunofluorescent double labeling of Hoxa2 with PDGFαR, sections were washed twice in PBS for 8 min, followed by a 2 min incubation in Digest-All (Zymed, San Francisco, CA). Subsequently, the sections were washed for 5 min in PBS and then blocked/permeabilized in 3% SM/0.1% TX for 20 min. They were then incubated overnight in primary antibodies diluted in 1% SM/0.03% TX. After two 8-min PBS washes, sections were incubated for 3 h in secondary antibodies (goat anti-rat IgG Alexa 594 (1:400) and goat anti-rabbit IgG Alexa 488 (1:200) [Invitrogen, Burlington, ON]), which were also diluted in 1% SM/0.03% TX. This was followed by an 8-min incubation in Hoechst fluorescent nuclear dye. Following these

incubations the sections were washed twice for 8 min each in PBS. Finally, the sections/coverslips were mounted with Prolong® Gold antifade reagent.

5.3.3 Plasmid construction and transfection

The protocol described below was conducted by a former MSc student Ms. Monica Wang (MSc Thesis 2007).

The sense cDNA of *Hoxa2* was amplified from the pRSV-Hoxa2 vector via PCR utilizing the Hoxa2 primers listed in Table 5.1. The PCR product was then double digested with *SalI* and *ClaI* and cloned into the *SalI*/*ClaI* site of the pTRE-2hyg2-Myc vector (Clontech, Mountain View, CA). The construct was confirmed by DNA sequence analysis.

All plasmids used for transfection were purified utilizing the EndoFree Plasmid Maxi Kit (Qiagen, Mississauga, ON). After purification, 30 µg of the pTet-off vector (kind gift of Dr. Peter Dickie, University of Alberta) was linearized with *HindIII*, followed by phenol extraction and ethanol precipitation. Linearized plasmid (10 µg) was then added to 30 µl of 2.5 M $CaCl_2$ and made up to a total volume of 150 µl with sterile water. The DNA-$Ca_3(PO_4)_2$ precipitate was prepared by adding the above mixture dropwise to an equal volume of 2x HEPES-buffered saline while vortexing. After 15 min, this mixture was then slowly added to the growth medium [DMEM supplemented with 50 µg/ml apo-transferrin, 10 pg/ml D-biotin, 50 ng/ml sodium selenite, 5 µg/ml insulin, 1% antibiotic/antimycotic and 30% B104 conditioned medium] in a 60 mm dish, which had been plated the previous day with CG4 cells and was 80% confluent. The cells were incubated with the DNA precipitate in a humidified incubator in 5% CO_2 at 37°C for 12 h. The cells were then rinsed with PUCKs [0.1% glucose, 0.8% sodium chloride, 0.04% potassium chloride, 0.006% potassium phosphate, 0.005% sodium phosphate and 0.002% phenol red in triple distilled water] and maintained in growth medium for two days. Subsequently, stable CG4-tTA clones were selected in the presence of 300 µg/ml G418 (Sigma) in 100 mm dishes. The isolated clones were then examined for their ability to induce gene expression via transiently transfecting the cells with the control vector pTRE-2hyg2-Myc-Luc (Clontech, Mountain View, CA) utilizing the calcium phosphate precipitate method. The clone exhibiting the highest luciferase activity was chosen for the subsequent transfection. As such, the CG4-tTA cells were plated onto 100 mm dishes

Table 5.1 Primers utilized for PCR analysis

Gene	Forward Primer	Reverse Primer
β-actin[a]	5′-ATTGTAACCAACTGGGACG-3′	5′-TTGCCGATAGTGATGACCT-3′
Hoxa2[d]	5′-ATCGATAGAAGGCCATGAATTACG-3′	5′-TCGTCGACTTAGTAATTCAGATGC-3′
Hoxa2*	5′-CTGGATGAAGGAGAAGAAGGC-3′	5′-CGGTTCTGAAACCACACTTTC-3′
MBP[b]	5′-CTATAAATCGGCTCACAAGG-3′	5′-AGGCGGTTATATTAAGAAGC-3′
PST[c]	5′-ACTGAGGAGCACCAAGAGACGC-3′	5′-CCATGAAGGCAGGAATCCAAAGG-3′

a Hu et al. (2004)
b www.hcnr.med.harvard.edu/programs/atrc/reagent_details/MBP%20Rat.pdf
c Soares et al. (2000); PST-Polysialyltransferase
d Tan et al. (1992)
* designed by Dr. Shaoping Ji; utilized for RT-PCR analysis, Appendix B

at a density of 8 x 10^5 cells/dish two days prior to the transfection. The cells were transfected with the recombinant plasmid pTRE-2hyg2-Myc-Hoxa2-S via electroporation, which was carried out using the Gene Pulser and a 0.4 cm electroporation cuvette (Bio-Rad Laboratories, Hercules, CA). The cells were harvested and collected in electroporation medium [1:1 HEPES-buffered saline and growth medium containing twice the concentration of insulin] at a concentration of 5 x 10^6 cells/ml. Subsequently, 20 µg of DNA was diluted in 20 µl of sterile water and added to 0.78 ml of cell suspension. Electroporation was performed at RT with a charge of 0.34 kV at a capacitance of 250 µF. Following electroporation the cells were allowed to sit for 10 min prior to plating them in recovery medium [DMEM supplemented with 2 mM sodium pyruvate, 5% FBS, 5 µg/ml insulin, and 1% antibiotic/antimycotic] onto poly-D-lysine-coated culture dishes. After 2 h the medium was then changed to growth medium. Subsequently, stable clones were selected in the presence of 200 µg/ml Hygromycin B (Sigma, ON) and 100 µg/ml G418.

5.3.4 CG4 cells

Hoxa2-S and WT CG4 cells were initially expanded in growth medium. Subculturing involved rinsing the cells first with PUCKs [Section 5.3.3, pg. 82] followed by an approximately 20 min incubation in harvesting medium [EDTA and PUCKs (1:1) supplemented with 25 mM glucose, 2 mM sodium pyruvate, 10 mM HEPES, 1.25 µg/ml insulin, and 1% antibiotic/antimycotic]. The cells were then collected and centrifuged at 800 rpm for 10 min. Subsequently, they were resuspended and plated in recovery medium. At the beginning of the trial, both Hoxa2-S and WT CG4 cells were plated at a density of 100,000 cells/well onto poly-D-lysine-coated wells or coverslips in separate 24-well plates. Following cell attachment, the recovery medium was changed to a differentiation medium. This medium was formulated similar to the growth medium [Section 5.3.3, pg. 82] except that it lacked B104 conditioned medium and, instead, contained 2% FBS. Subsequently, the cells were maintained in culture for either one or four days, at which time a portion of the wells containing Hoxa2-S and WT CG4 cells were processed.

5.3.5 Immunocytochemistry

Immunocytochemistry was conducted utilizing the following primary antibodies:

A2B5 (1:100, hybridoma; ATCC), O4 (1:1000, Cedarlane Laboratories Ltd, Burlington, ON), anti-GalC (1:100, Ranscht et al., 1982), anti-GFAP (1:800, Dako Canada Inc., Mississauga, ON), anti-PSA-NCAM (1:100, concentrate, Developmental Studies Hybridoma Bank), and anti-bromodeoxyuridine (BrdU) (1:400, Sigma-Aldrich, Oakville, ON). Cells were labeled for either A2B5/GalC or O4 utilizing the same protocol. These markers are used to identify cells at different stages of OG development [Figure 2.4]. The monoclonal antibody A2B5 is used to recognize a complex ganglioside expressed on the surface of OPCs (Abney et al. 1983; Eisenbarth et al. 1979). The monoclonal antibody O4 recognizes surface epitopes expressed by pro-OGs (Bansal et al. 1989). GalC, the major galactosphingolipid of myelin, marks pre-myelinating OGs (Ranscht et al. 1982). The cells were first fixed for 10 min with 4% formaldehyde after which they were washed two times for 5 min each with PBS. This was followed by two 30-min incubations: first in the primary antibodies and then in the secondary antibodies [goat anti-mouse IgM μ-chain specific Alexa 594 (1:400, A2B5/O4; Invitrogen, Burlington, ON) and goat anti-mouse IgG γ-chain specific FITC (1:50, GalC/MOG; BIO/CAN Scientific, Mississauga, ON)]. The nuclei of all cells were then stained with a 10-min incubation in Hoechst dye (Sigma-Aldrich, Oakville, ON). Following these incubations the cells were washed twice with PBS for 5 min each. Finally, the cells/coverslips were mounted utilizing ProLong® Gold antifade reagent (Invitrogen, Burlington, ON).

Double labeling cultures for PSA-NCAM/GFAP was conducted as described above with a few modifications. First, the cells were initially stained for PSA-NCAM and then GFAP. Second, the anti-GFAP antibody was diluted in 1% SM/0.03% TX instead of PBS. Finally, the secondary antibodies utilized were goat anti-mouse IgM μ-chain specific Alexa 594 (1:400, PSA; Invitrogen, Burlington, ON) and goat anti-rabbit IgG Alexa 488 (1:200, GFAP; Invitrogen, Burlington, ON).

For staining proliferative cells, BrdU (Sigma-Aldrich, Oakville, ON) at a final concentration of 1 μM was added to the culture medium 1.5 h prior to processing. Subsequently, the cells were fixed with 4% formaldehyde for 10 min followed by two 5 min washes with PBS. The cells were then incubated at 37ºC for 30 min in 2N HCl. This was followed by two 5 min PBS washes and a 10 min block/permeabilization in 3% SM/0.1% TX. The cells were then incubated for 1 h in primary antibody, 30 min in the

secondary antibody (goat anti-mouse IgG γ-chain specific FITC [1:50, BIO/CAN, Mississauga, ON]), and finally 20 min in a nuclear fluorescent Hoechst counterstain. Two 5 min PBS washes were done between, as well as following these incubations. The cells/coverslips were subsequently mounted in ProLong® Gold antifade reagent.

The percentage of cells that were stained with a particular antibody was determined by counting the immunoreactive and Hoechst$^+$ cells on micrographs taken from every third field at a magnification of 20 X for a total of 25 fields utilizing the ImagePro® Express 5.1 software (Olympus Canada Inc., Markham, ON). Most of this data were then subjected to statistical analysis utilizing a two-way analysis of variance (ANOVA) to determine the significant main effects and a Bonferroni's *post hoc* test to determine statistical significance ($p<0.05$). In the case of BrdU, the statistical analysis utilized was the Mann-Whitney test.

5.3.6 Reverse transcriptase-polymerase chain reaction

Samples were collected for RNA isolation via aspirating the culture medium and subsequently lysing the cells with 350 µl of Buffer RLT. Lysed cells were collected in 1.5 ml Eppendorf tubes, vortexed for 1 min, and then stored at -80ºC until they were processed. Total RNA was extracted utilizing the RNeasy® Mini kit (Qiagen Inc., Mississauga, ON) as per manufacturer's instructions. cDNA was subsequently obtained utilizing SuperScript™ Reverse Transcriptase (Invitrogen, Burlington, ON) according to the manufacturer's instructions with the following modification: after the addition and gentle mixing of SuperScript™ Reverse Transcriptase the tubes were incubated at 25ºC for 10 min, 37ºC for 1.5 h, and finally 70ºC for 15 min. The EconoTAQ™ DNA Polymerase (VWR, West Chester, PA) kit was subsequently utilized for PCR analysis. As such, the following chemicals were added to the PCR tube: 2 µl cDNA, 5 µl 10X Buffer, 1.5 µl primer 1 and 2 (10 µM), 1 µl of each dNTP, 1 µl TAQ polymerase, sterile distilled water to 50 µl. The oligonucleotide DNA primers utilized are listed in Table 5.1. PCR was performed in a PTC-100™ thermal cycler. After an initial denaturation step for 1.5 min at 94°C, 30 cycles were carried out with each cycle consisting of a denaturation (45 s at 94°C), annealing (1 min at 55°C) and elongation (1 min at 72°C) step. This was followed by a further elongation step for 4 min at 72°C. PCR products were analyzed by electrophoresis on 1% agarose gels using 1xTAE as the running buffer. The resulting

bands, which were visualized with ethidium bromide, were photographed with the AlphaImager™ Gel Imaging System.

5.3.7 Western blot analysis

Culture medium was aspirated from individual wells after which 200 µl of RIPA buffer [150 mM NaCl, 0.5 ml TX, 0.01% SDS, 0.1% sodium deoxycholate, 10 mM Tris, 5 mM EDTA in triple distilled water] was added to lyse the cells. Cell lysate was subsequently collected in 1.5 ml Eppendorf tubes and stored at -80°C until they were processed. Protein quantification was conducted utilizing the DC Protein Assay (Bio-Rad Laboratories Ltd., Mississauga ON) as per manufacturer's instructions. Subsequently, equal amounts of protein for each sample were separated on a 10% polyacrylamide-SDS gel and transferred to a PolyScreen® PDVF membrane (PerkinElmer, Boston, MA). The membrane was subsequently incubated in 3% SM in PBS overnight in order to block non-specific binding. This was followed by two 1 h incubations, first in primary antibody (anti-actin-1:1000, Developmental Studies Hybridoma Bank; anti-Hoxa2- 1:1000, Hao et al. (1999); or anti-NCAM- 1:1500, kind gift of ENKAM Pharmaceuticals A/S, University of Copenhagen, Denmark) and then, subsequently, in secondary antibody (goat anti-mouse IgM horseradish peroxidase conjugate-1:750, Cedarlane Laboratories Ltd., Burlington ON; or goat anti-rabbit IgM horseradish peroxidase conjugate- 1:1500, Bio-Rad Laboratories Ltd., Mississauga ON). Following these incubations, the membrane was washed three times for 20-30 min each with 0.08% Tween-20 in PBS. Subsequently, the membrane was treated briefly with a chemiluminescence agent (PerkinElmer) and then exposed to X-ray film. The resulting bands were subsequently visualized on X-ray film.

5.4 Results

5.4.1 *Hoxa2* is expressed in multiple regions of the embryonic telencephalon

To examine *Hoxa2* expression in the telencephalon, *in situ* hybridization histochemical analysis was conducted on E13.5 (C18/T20) transverse brain sections. The results show that *Hoxa2* is expressed in the ventricular zone of the MGE, LGE, and

Figure 5.1 *Hoxa2* and *Hoxd1* are expressed in the embryonic murine telencephalon.

Transverse sections of E13.5 (C18/T20) CD1 murine brains were subjected to *in situ* hybridization histochemical analysis for *Hoxa2* (A,B) [figure (B) is a higher magnification of figure (A)] and *Hoxd1* (C). *Hoxa2* mRNA expression is evident throughout the telencephalon, being more intense in the dorsal telencephalon (P) and the LGE than in the MGE (A,B). Similar to *Hoxa2*, *Hoxd1* is expressed throughout the E13.5 (C18/T20) telencephalon (C). Abbreviations: SVZ-subventricular zone, VZ-ventricular zone. Bar = 100 μm except for (A) where bar = 200 μm. [taken from Louise Wolf's PhD Thesis 2004]

dorsal telencephalon, as well as the subventricular zone of the LGE [Figure 5.1A,B]. As *Hoxd1* exhibits a similar expression profile to *Hoxa2* in the diencephalon (Wolf et al. 2001), its expression was also examined in this region. Similar to *Hoxa2*, *Hoxd1* mRNA is expressed throughout the E13.5 (C18/T20) telencephalon [Figure 5.1C].

5.4.2 Olig2 and Pax6 telencephalic expression profiles are not altered in the absence of *Hoxa2*

Hoxa2+/+, *Hoxa2*+/- and *Hoxa2*-/- telencephalic sections were analyzed for the expression of Olig2 and Pax6, two TFs whose expression profiles are limited to either the ventral or dorsal telencephalon, respectively. *Hoxa2*+/- and *Hoxa2*+/+ mice were used interchangeably to represent the presence of *Hoxa2*, since Olig2 and Pax6 expression profiles were found to be similar in both genotypes. The results of this analysis show that Olig2 is primarily expressed in the neuroepithelium of the MGE and LGE in both the presence and absence of *Hoxa2* [Figure 5.2A,B]. Similarly, regardless of genotype, Pax6 is expressed in the neuroepithelium of the dorsal telencephalon [Figure 5.2C,D].

5.4.3 Telencephalic PDGFαR expression is unaltered in the absence of *Hoxa2*

In order to determine if Hoxa2 is expressed by OPCs in the telencephalon, double immunofluorescent analysis was conducted on E18 (T25) coronal telencephalic sections. This analysis revealed that Hoxa2 is expressed by PDGFαR$^+$ OPCs in the corpus callosum [Figure 5.3A-C] and cerebral cortex [Figure 5.3F-H]. Although its expression is more abundant in the cerebral cortex, only a small subset of the PDGFαR$^+$ cells express Hoxa2 in both regions. In turn, Hoxa2's expression in the telencephalon is not limited to OGs as evident by the fact that it is expressed by cortical cells that are PDGFαR$^-$ [Figure 5.3]. Subsequently, immunofluorescent analysis was conducted to determine whether PDGFαR expression is affected in either of these areas in the absence of *Hoxa2*. The results showed that PDGFαR$^+$ cells are evident in both the cerebral cortex [Figure 5.3I,J] and the corpus callosum [Figure 5.3D,E] regardless of genotype.

5.4.4 There are significantly fewer O4$^+$ and GalC$^+$ cells in CG4 cells over-expressing *Hoxa2*

In order to examine Hoxa2's role in OG differentiation, CG4 cells, established from perinatal rat cortical OPCs (Louis et al. 1992), were stably transfected with a Tet-off

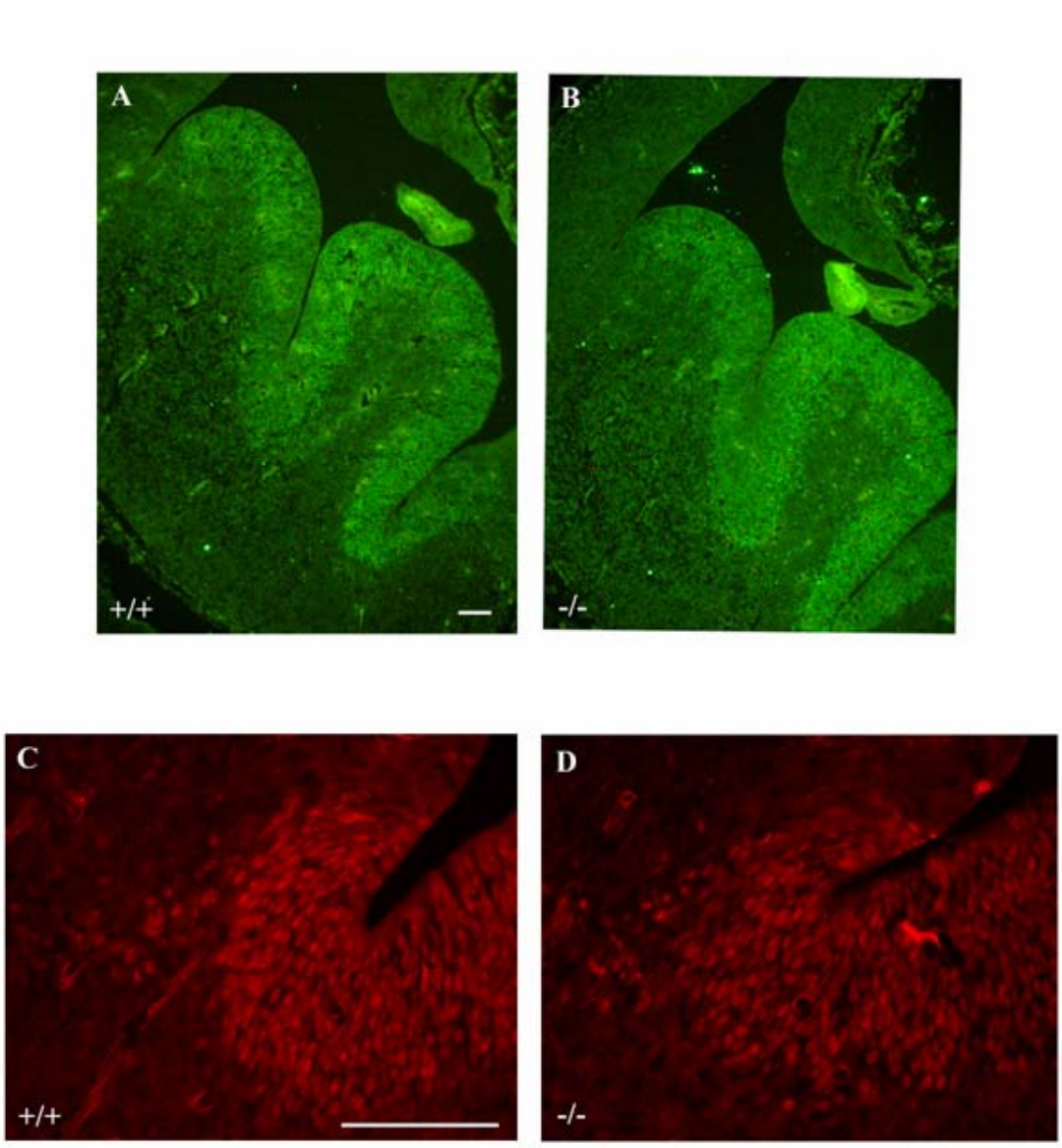
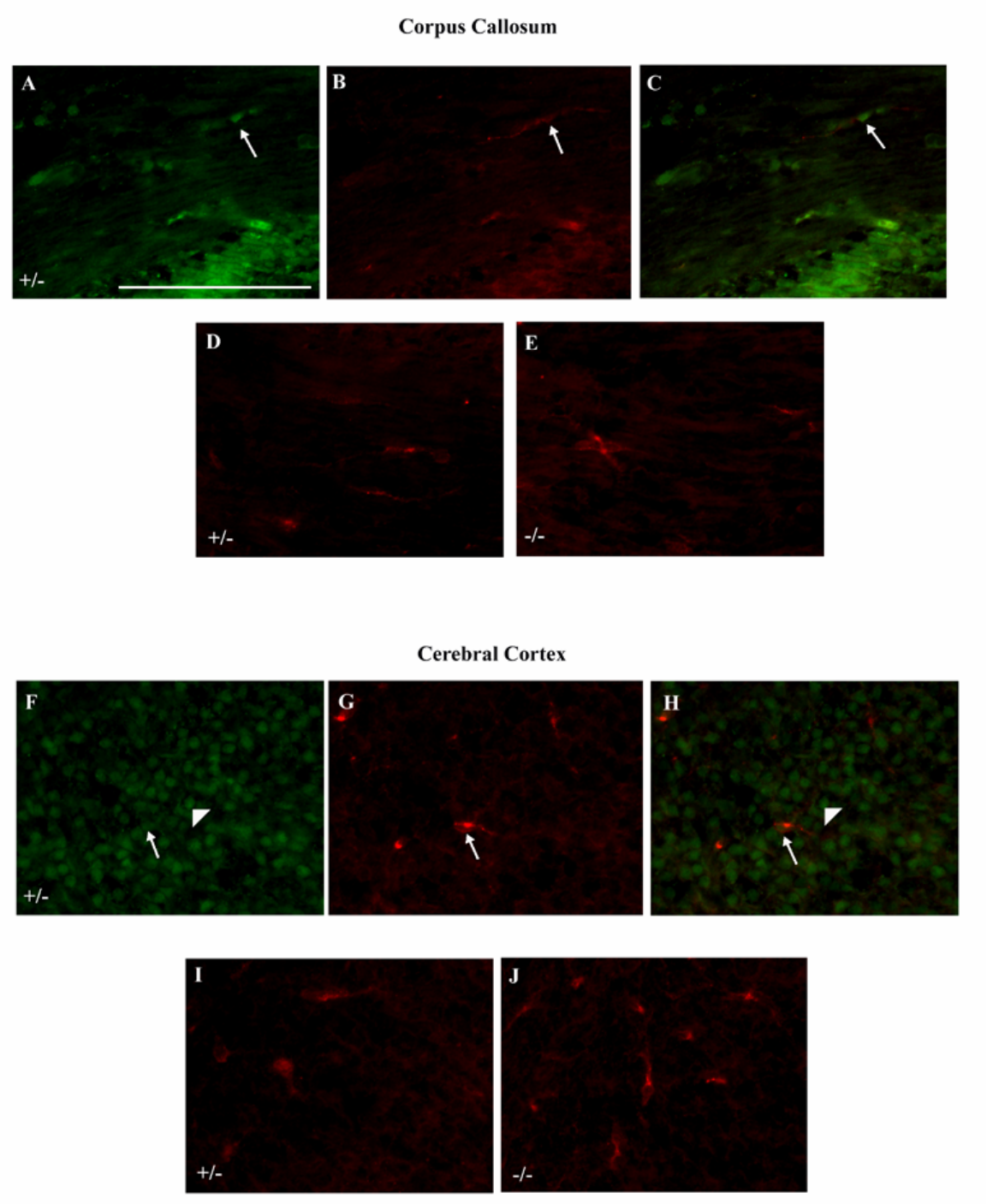


Figure 5.2 Olig2 and Pax6 exhibit similar expression profiles in *Hoxa2*+/+ and *Hoxa2*-/- mice.

E14.25 (C19+/T21) coronal telencephalic sections were immunolabeled for Olig2 (A,B) and Pax6 (C,D) as described in Materials and Methods. The expression profiles of these TFs were found to be similar both in the presence (A,C) and absence (B,D) of *Hoxa2*. Scale bars = 100μm.

Figure 5.3 PDGFαR expression in the telencephalon of E18 (T25) *Hoxa2+/-* and *Hoxa2-/-* mice.

Coronal telencephalic sections from E18 (T25) *Hoxa2+/-* (A-D, F-I) and *Hoxa2-/-* (E, J) mice were immunolabeled for Hoxa2 (A, F) and/or PDGFαR (B,D,E,G,I,J) as described in Materials and Methods. A small population of PDGFαR^{+} cells was found to express Hoxa2 (arrows) in both the corpus callosum (A-C) and cerebral cortex (F-H). The arrowhead in (H) demarcates a Hoxa2^{+}/ PDGFαR^{-} cell. [The first two micrographs in the first and third rows (A,B and F,G) represent individual filters (fluorescein- A,F; rhodamine-B,G) of the same region, whereas the third micrograph (C,H) represents a merging of these filters.] The expression profile of PDGFαR in these telencephalic regions was found to be similar in the presence (D,I) and absence (E, J) of *Hoxa2*. Analyses were conducted on sections obtained from two *Hoxa2*+/- and *Hoxa2*-/- embryos. Scale bar = 100μm.


Corpus Callosum
A
B
C
+/-
D
E
+/-
-/-
Cerebral Cortex
F
G
H
+/-
I
J
+/-
-/-

vector containing a Hoxa2-S construct. Subsequently, both WT and Hoxa2-S CG4 cells were grown in differentiation medium for either one or four days, at which point they were processed via immunocytochemistry for the following OG markers: A2B5, O4, and GalC. The data obtained from this experiment were then subjected to a two-way ANOVA with the data from each OG marker being analyzed separately for statistical significance. Multiple *post hoc* comparisons were then performed utilizing Bonferroni's multiple comparison post-test. The two-way ANOVA revealed significant main effects of culture type [A2B5: $F(1,8)=14.01$, $p=0.0057$; O4: $F(1,8)=20.40$, $p=0.0020$; GalC: $F(1,8)=18.04$, $p=0.0028$] and time [A2B5: $F(1,8)=20.25$, $p=0.0020$; O4: $F(1,8)=22.23$, $p=0.0015$; GalC: $F(1,8)=8.93$, $p=0.0174$] for all three OG markers. However, regardless of the marker, it failed to reveal a significant interaction between these factors [A2B5: $F(1,8)=1.469$, $p>0.05$; O4: $F(1,8)=1.565$, $p>0.05$; GalC: $F(1,8)=2.234$, $p>0.05$]. Subsequently, Bonferroni's post-test revealed that at one day in culture, significantly more $A2B5^+$ cells are found in the Hoxa2-S cultures in comparison to WT. It also revealed that the Hoxa2-S cultures contain significantly more $A2B5^+$ cells at one day compared to four days in culture [Figure 5.4A]. An additional finding was that after four days in culture the percentage of $O4^+$ and $GalC^+$ cells is significantly lower in the cells over-expressing *Hoxa2* in comparison to WT CG4 cells [Figure 5.4B,C]. In agreement with this data, RT-PCR analysis suggests that *MBP* expression is lower in Hoxa2-S cultures when compared to WT [Figure 5.4D].

5.4.5 The median percentage of $BrdU^+$ cells is similar in Hoxa2-S and wild-type cultures

In order to determine if *Hoxa2* over-expression affects CG4 cell proliferation, immunocytochemical analysis for BrdU was conducted on WT and Hoxa2-S CG4 cells at one day. The results of this analysis failed to reveal a significant difference [$p>0.05$] between WT CG4 cells and those over-expressing *Hoxa2* with regards to the median percentage of BrdU-immunoreactive cells [Table 5.2].

5.4.6 Over-expression of *Hoxa2* in CG4 cells results in significantly fewer PSA-$NCAM^+$ cells

PSA-NCAM was examined as a potential downstream target of Hoxa2 in CG4 cells via conducting immunocytochemical analysis on Hoxa2-S and WT CG4 cells at

Figure 5.4 Over-expression of *Hoxa2* in CG4 cells results in significantly fewer pro-oligodendrocytes and pre-myelinating oligodendrocytes.

(A-C) After either one (1d) or four days (4d) in culture, both Hoxa2-S and WT CG4 cells were processed via immunocytochemistry for A2B5, O4, and GalC as described in Materials and Methods. The histogram bars represent the mean of the percentages obtained from three individual coverslips, which were treated identically. The error bars represent the standard error of the mean. *Post hoc* comparisons were performed with Bonferroni's multiple comparison post-test. This post-test revealed that the percentage of $A2B5^+$ cells is significantly higher ($p<0.05$; bracket) in the Hoxa2-S cultures than WT at 1 day. In addition, a significant decrease ($p<0.05$; bracket) in the percentage of A2B5-immunoreactive cells is seen between 1d and 4d (A). In comparison to A2B5, the percentage of $O4^+$ (B) and $GalC^+$ (C) cells is significantly lower ($p<0.05$; bracket) in the Hoxa2-S cultures compared to WT at four days. As would be expected with differentiating cultures, the percentage of O4-immunoreactive cells is significantly higher ($p<0.05$; bracket) in the 4d WT cultures in comparison to the 1d (B).

(D) After four days in culture, a subset of the cells were collected and processed via RT-PCR for *MBP* and *β-actin* as described in Materials and Methods. Given similar amounts of cDNA, as shown by *β-actin*, *MBP* mRNA expression appears to be weaker in Hoxa2-S cultures in comparison to WT.

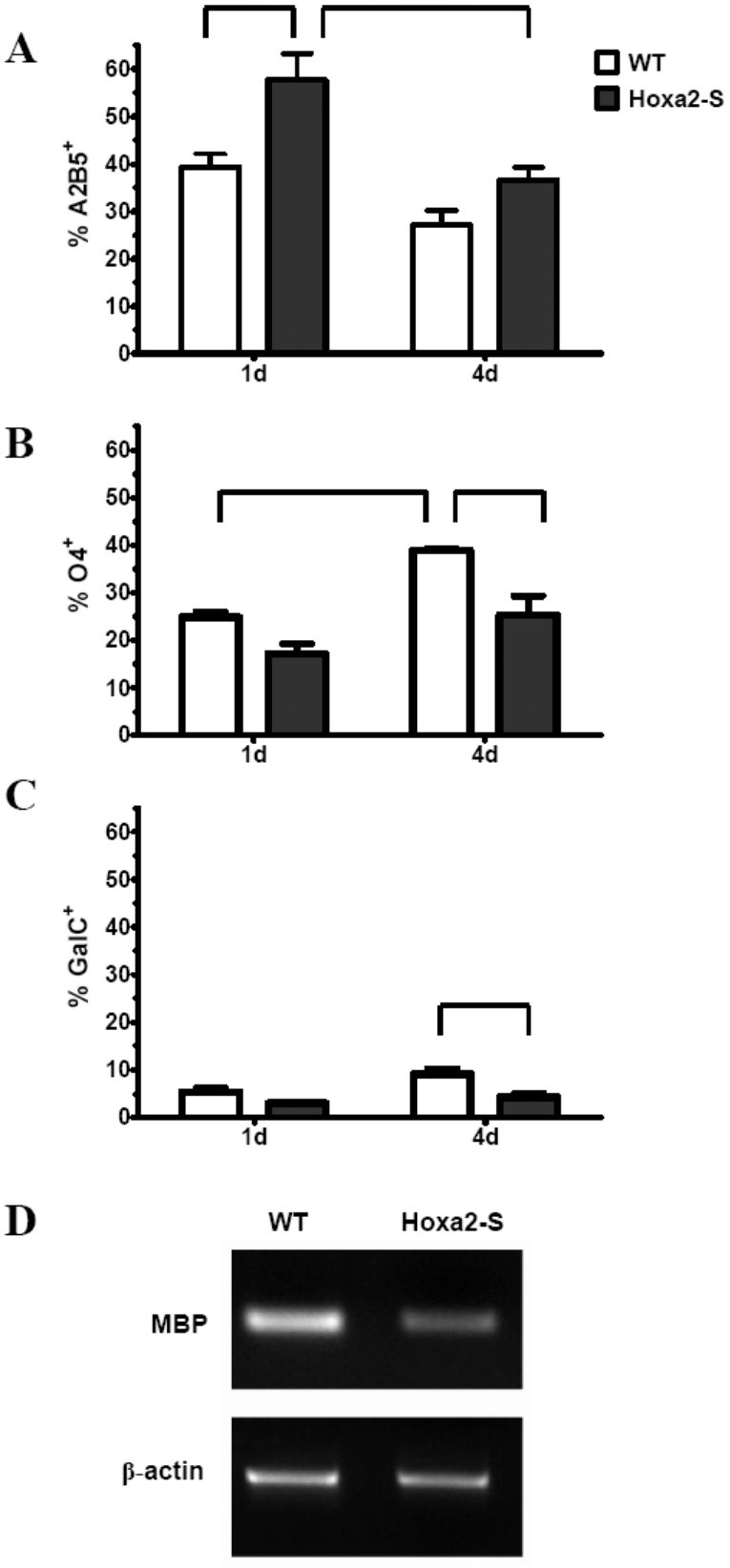

A
WT
Hoxa2-S
% A2B5+
60
50
40
30
20
10
0
1d
4d
B
% O4+
60
50
40
30
20
10
0
1d
4d
C
% GalC+
60
50
40
30
20
10
0
1d
4d
D
WT
Hoxa2-S
MBP
β-actin

Table 5.2 The median percentage of proliferating cells is similar in Hoxa2-S and wild-type CG4 cultures.

	Median % Coverslip #1	**Median % Coverslip #2**	**Median % Coverslip #3**
WT CG4	40.7	47.1	43.8
Hoxa2-S CG4	45.9	45.9	37.8

WT and Hoxa2-S CG4 cells were grown in differentiation medium for one day at which point they were processed via immunocytochemistry for BrdU as described in Materials and Methods. Statistical significance was determined utilizing a Mann-Whitney test. The median percentage of $BrdU^+$ cells was found to be similar regardless of the culture type [$p>0.05$].

both one and four days in culture. The data obtained from this experiment were then subjected to a two-way ANOVA, as well as a Bonferroni's multiple comparison post-test. The two-way ANOVA revealed significant main effects of culture type [$F(1,8)=406.4$, $p<0.0001$] and time [$F(1,8)=106.8$, $p<0.0001$], as well as a significant interaction between these factors [$F(1,8)=48.78$, $p=0.0001$]. Subsequently, Bonferroni's post-test showed that there are significantly fewer PSA-NCAM-immunoreactive cells in Hoxa2-S cultures compared to WT, regardless of the time *in vitro*. In addition, it showed that the percentage of PSA-NCAM$^+$ cells is significantly increased in WT CG4 cells at four days versus one day in culture [Figure 5.5].

As PSA-NCAM is a post-translational modification of the NCAM molecule, western blot analysis was conducted on the cultures at four days to determine if NCAM expression is also reduced in CG4 cells over-expressing *Hoxa2*. Furthermore, RT-PCR analysis was also conducted to determine whether the expression of *PST*, which is the enzyme responsible for adding polysialic acid units to the NCAM molecule in oligodendroglial cells (Stoykova et al. 2001), is affected in the Hoxa2-S cultures. The results suggest that both NCAM and *PST* expression is reduced in Hoxa2-S cultures compared to WT [Figure 5.6].

5.5 Discussion

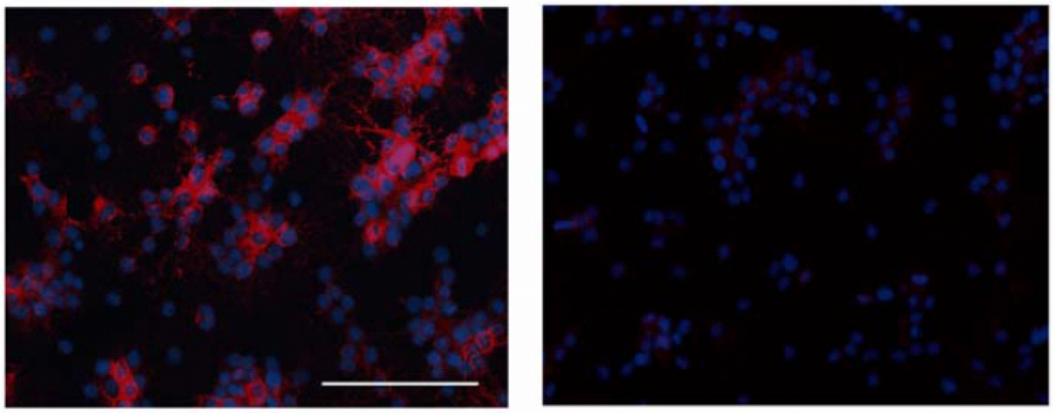
The TF Hoxa2 is expressed in various regions and cell types in the CNS (Hao et al. 1999; Nicolay et al. 2004a; Tan et al. 1992; Wolf et al. 2001). However, current knowledge regarding its function in the CNS is limited to early stages of development, such as dorsal-ventral and anterior-posterior patterning, as well as neuronal development (Davenne et al. 1999; Gavalas et al. 1997). Our laboratory is interested in investigating Hoxa2's function at later stages of CNS development, specifically during oligodendrogenesis.

In the present study, we have investigated the expression and function of Hoxa2 in the telencephalon. Expression analysis of *Hoxa2* in the E13.5 telencephalon revealed that it was expressed in three regions known to give rise to OPCs [Figure 5.1A,B]. Hence, this suggested that it could play a role in OG specification in one or more of these regions. As there are no known markers that can distinguish between the OPCs arising from these distinct telencephalic domains, we could not address this issue directly.

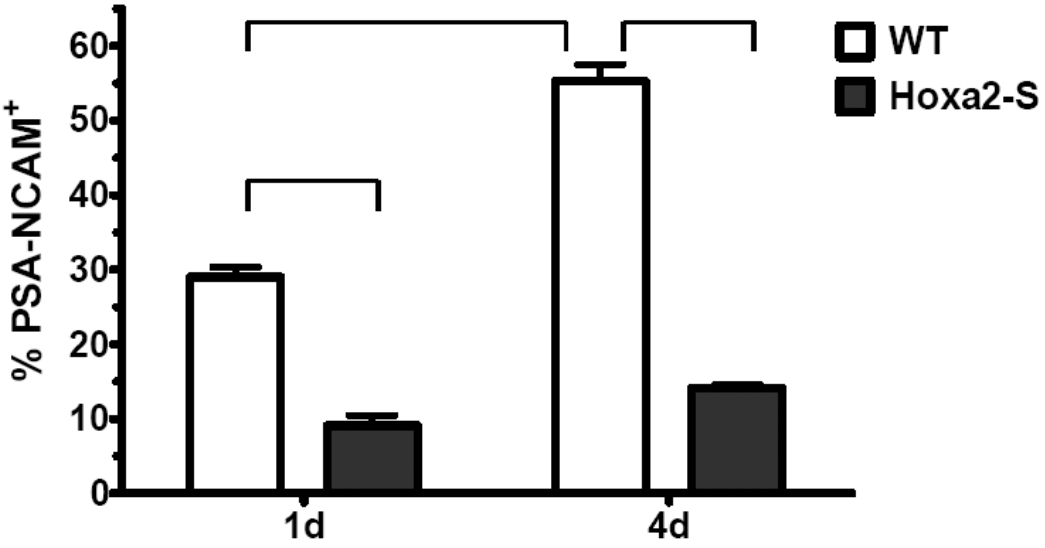
Figure 5.5 The percentage of PSA-NCAM$^+$ cells is significantly reduced in the Hoxa2-S CG4 cultures in comparison to wild-type.

WT and Hoxa2-S CG4 cells were grown in differentiation medium for one day at which point they were processed via immunocytochemistry for PSA-NCAM. The top row (A) contains representative micrographs illustrating PSA-NCAM expression in WT and Hoxa2-S cultures at 4d [Scale bar = 100 μm]. The histogram bars represent the mean of the percentages obtained from three individual coverslips, which were treated identically. In addition, the error bars represent the standard error of the mean. *Post hoc* comparisons were performed with Bonferroni's multiple comparison post-test. This post-test revealed that the percentage of PSA-NCAM$^+$ cells is significantly lower ($p<0.05$; brackets) in Hoxa2-S cultures in comparison to WT at both 1d and 4d. Furthermore, whereas there is a significant increase ($p<0.05$; bracket) in the percentage of immunoreactive cells in the WT cultures between 1d and 4d, the same is not found in the Hoxa2-S cultures.

A



B



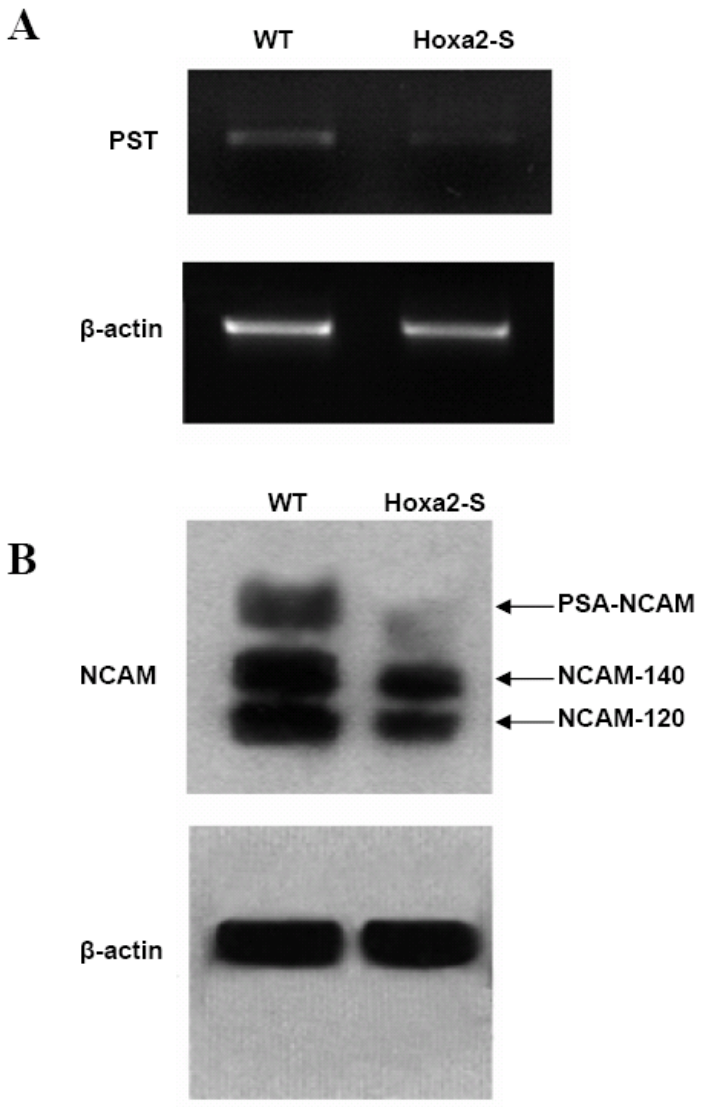


Figure 5.6 Over-expression of *Hoxa2* may reduce PSA-NCAM expression via multiple mechanisms.

Hoxa2-S and WT CG4 cells were grown in differentiation medium for four days. A subset of the cells were then collected and processed via RT-PCR for *PST* and *β-actin* (A). At the same time another portion of the wells was collected and processed by Western blot analysis of NCAM and β-actin (B). Given similar amounts of cDNA (A) or protein (B), the levels of *PST* (A) and NCAM (B) expression appears to be weaker in the Hoxa2-S cultures than the WT. (The RT-PCR and Western blot analysis was done in triplicate.)

Instead, we examined OG specification in the ventral telencephalon of *Hoxa2* transgenic knockout mice via analyzing the expression of the TF Olig2. This TF, which is expressed in the ganglionic eminences (Takebayashi et al. 2000), was chosen based on the finding that in *Olig2* mutant mice OPCs are largely absent in the forebrain except for a focal region in the cortex (Lu et al. 2002). As the results of our analysis failed to reveal any differences in Olig2's expression profile [Figure 5.2], it suggests that OG specification in the ventral telencephalon occurs normally in the absence of *Hoxa2*. However, as there is a focal region of the telencephalon that still contains OPCs in *Olig2* mutant mice (Lu et al. 2002), further research will need to be conducted as more information becomes known regarding the regulation of ventral and dorsal OG specification in this region.

In comparison to its ventral counterpart, information regarding the transcriptional control of OG specification in the dorsal telencephalon is currently lacking. However, there is evidence to suggest that it is regulated differently than that of the ventral telencephalon. Although Olig2 is expressed by dorsal OPCs in the telencephalon, it is not required for their specification (Yue et al. 2006). As a result, it was decided to analyze the expression of Pax6 in the telencephalon of *Hoxa2* transgenic knockout mice in order to determine if dorsal telencephalic patterning and, hence, development was affected in the absence of *Hoxa2*. This TF was chosen because its mRNA expression is predominantly found in the neuroepithelium of the dorsal telencephalon (Puelles et al. 1999; Walther and Gruss 1991). Furthermore, recent research indicates that Pax6 functions in dorsal telencephalic development via repressing the expression of TFs normally found in the ventral telencephalon, as well as preventing premature differentiation and depletion of the cortical progenitor pool (Quinn et al. 2007). The results of this analysis revealed that Pax6 is expressed similarly regardless of genotype [Figure 5.2]. Therefore, this suggests that dorsal telencephalic development and, hence, oligodendrogenesis occurs normally in the absence of *Hoxa2*. This, however, will have to be verified as more information is obtained regarding the transcriptional regulation of OG specification in this region.

Subsequent double immunofluorescent analysis of Hoxa2 with PDGFαR showed that Hoxa2 is expressed by PDGFαR$^+$ OPCs in the embryonic telencephalon [Figure 5.3]. These results support our previous immunocytochemical analysis which showed that

Hoxa2 is expressed by A2B5-immunoreactive cells in primary mixed glial cultures obtained from newborn cerebral hemispheres (Nicolay et al. 2004a) [Section 3.4.1, pg. 51]. Interestingly, Hoxa2's expression profile during OG development *in vitro* is reminiscent of that seen with several other TFs, such as Sox5 and Sox6, which have been shown to inhibit OG differentiation (Stolt et al. 2006). Based on this information we postulated that Hoxa2 could play a role in this process. As our *Hoxa2* transgenic knockout mice die shortly after birth, which is before $GalC^+$ OGs are known to arise in the telencephalon, we could not examine OG differentiation in these mice *in vivo* (Gard and Pfeiffer 1989). Furthermore, as several additional Hox TFs have been shown to be expressed by oligodendroglial cells in primary mixed glial cultures obtained from the cerebral hemispheres of newborn mice (Nicolay et al. 2004b) [Section 4.4, pg. 72; Appendix B], these TFs could compensate for *Hoxa2* in its absence. As a result, it was decided to over-express *Hoxa2* in the CG4 oligodendroglial cell line in order to determine if Hoxa2 could play a role in their differentiation. The reason we chose to utilize a cell line instead of primary cultures was due to its advantages regarding accessibility and increased rate of differentiation. This cell line was established from primary cultures of oligodendroglial cells obtained from the perinatal rat cerebral cortex (Louis et al. 1992). These cells not only display numerous characteristics of normal oligodendroglial cells including morphological and antigenic phenotypes [Appendix B], but they also respond to similar mitogens (Louis et al. 1992).

In this experiment WT and Hoxa2-S CG4 cells were grown in differentiation medium for either one or 4 days at which point immunocytochemical analysis was conducted to determine what stage of development the cells were at. The results show that at one day the only significant difference between the cultures was in regards to the percentage of $A2B5^+$ cells, being significantly higher in Hoxa2-S cultures compared to WT [Figure 5.4A]. The reason for this effect is uncertain; however, it could be due to several factors. For instance, it could be due to the fact that *Hoxa2*-over-expression affects CG4 proliferation. However, as the median percentage of $BrdU^+$ is similar in both Hoxa2-S and WT cultures [Table 5.2], this is unlikely. As CG4 cells can give rise to both OGs and type 2 astrocytes, which both express A2B5, the higher percentage of $A2B5^+$ cells at one day in the Hoxa2-S cultures could reflect an enhancement of astrocyte

development in these cultures compared to WT. However, as the expression of the astrocytic marker GFAP is negligible in all cultures regardless of time, this appears unlikely.

The results also showed that the percentage of $A2B5^+$ cells is significantly reduced in Hoxa2-S cultures at 4 days in comparison to one day [Figure 5.4A]. This finding would suggest that the cells are progressing through to later stages of development. It was somewhat surprising, therefore, to find that there are significantly fewer O4- and GalC-immunoreactive cells in the Hoxa2-S cultures at four days [Figure 5.4B,C]. These findings leave us with the question of why A2B5 expression is decreased in these *Hoxa2* over-expressing cells. As $A2B5^+$ cells may also co-express O4 the decrease could be due, in part, to the decrease in the percentage of $O4^+$ cells.

In an attempt to identify potential mechanisms by which Hoxa2 could affect OG differentiation, we conducted a literature search in hopes of discovering downstream targets of Hox TFs that are expressed by OGs. Based on this search we decided to focus on the cell surface glycoprotein NCAM. This molecule exists as three prominent protein isoforms (Barbas et al. 1988), which can undergo a variety of post-translational modifications. One such modification, glycosylation, can lead to the formation of the embryonic form of NCAM, known as PSA-NCAM (Rothbard et al. 1982). Interestingly, PSA-NCAM, which is expressed primarily by $A2B5^+$ cells (Trotter et al. 1989), has been implicated in inhibiting OG differentiation (Decker et al. 2000). As a result, PSA-NCAM expression was examined in WT and Hoxa2-S CG4 cultures after one and four days in differentiation medium to see if it was up-regulated in the Hoxa2-S cultures. However, contrary to our hypothesis, we find that the percentage of $PSA\text{-}NCAM^+$ cells is significantly lower in Hoxa2-S cultures when compared to WT regardless of the time in culture [Figure 5.5B]. This is somewhat surprising based on the findings of Decker et al. (2000), who report an increase in the percentage $O4^+$ cells following removal of the polysialic acid residue with endoN. Closer examination of their data, however, reveals that this effect is only evident under certain *in vitro* conditions (Decker et al. 2000). Furthermore, a similar experiment conducted by a different research group failed to reveal any differences in the percentage of $GalC^+$ cells in endoN-treated and control cultures (Zhang et al. 2004). Based on this information, as well as our results, it appears

that Hoxa2's effect on CG4 cell differentiation is independent of its effect on PSA-NCAM expression. Although Hoxa2's effect on PSA-NCAM expression is opposite to what was expected, distinct *Hox* genes are known to differentially affect NCAM promoter activity (Jones et al. 1993; Jones et al. 1992). Based on our western blot analysis, Hoxa2 appears to negatively affect PSA-NCAM expression, in part, via regulating NCAM expression.

In order to properly interpret these findings it should be kept in mind that the overall effect of *Hoxa2* over-expression in CG4 cells can be impacted by several factors. In particular, the degree of the effect may be limited by the availability of the co-factors Pbx and/or Prep/Meis. Furthermore, as *Hox* genes are known to undergo auto- and cross-regulation (Lampe et al. 2004; Tümpel et al. 2007), over-expressing *Hoxa2* in CG4 cells could impact its expression, as well as other *Hox* genes. Finally, the environmental factors at play in these cultures may impact the effect of *Hoxa2* over-expression on CG4 development. For example, as downstream effectors of the BMP signaling pathway can modulate the transcriptional activities of Hox TFs, Hoxa2's effect on CG4 development could be impacted by the presence or absence of BMPs (Li et al. 2006).

One additional finding from our experiment is that in WT cultures the percentage of PSA-NCAM$^+$ and A2B5$^+$ cells is increased and decreased, respectively, over time [Figure 5.4A, 5.5B]. Furthermore, examination of the morphology of the PSA-NCAM-immunoreactive cells revealed multipolar cells indicative of later stages of OG development [Figure 5.5A]. Hence, these findings contradict a previous study which found that PSA-NCAM is expressed primarily by OPCs (Trotter et al. 1989). The reason for this disparity is unknown; however, it could reflect differences between primary cultures and CG4 cells. Alternatively, it could reflect differences in PSA-NCAM expression in specific CNS regions, as the primary cultures utilized by Trotter et al. (1989) were obtained from the cerebellum whereas the CG4 cell line was established from the cerebral cortex (Louis et al. 1992). Further research will be needed to resolve this difference.

In addition to being implicated in OG differentiation, PSA-NCAM has been shown to be important in PDGF-directed OPC migration (Zhang et al. 2004). Hence, this suggests that Hoxa2 could negatively impact this trait in OPCs. Although

immunohistochemical analysis of E18 coronal telencephalic sections failed to reveal any noticeable differences in PDGFαR expression [Figure 5.3], other Hox TFs are found in the telencephalon which could potentially compensate for Hoxa2 in its absence. Hence, further research will be needed to address this issue.

In conclusion, our results support a role for Hoxa2 in OG differentiation. Furthermore, they suggest that Hoxa2 may affect additional aspects of OG development, since PSA-NCAM is involved in PDGF-directed OPC migration (Zhang et al. 2004).

Preamble to Chapter 6

The expression of *Hox* genes is enhanced by RA, which, in turn, can either inhibit or promote OG differentiation. Although the reason for these opposing roles is uncertain, examination of the experimental protocols utilized by different research groups reveals differences in age, CNS region, as well as RA concentration. As a result, RA's effect on oligodendrogenesis could be time- and/or concentration-dependent. This chapter is focused on determining which of these factors can impact RA's effect on oligodendroglial differentiation.

6.0 RETINOIC ACID'S EFFECT ON THE DIFFERENTIATION OF MYELINATING GLIAL CELLS IS IMPACTED BY CONCENTRATION AND TIME

6.1 Summary

1. RA can either inhibit or promote OG differentiation. Although the factors that dictate what effect RA has on OG differentiation is uncertain, they may include RA concentration and/or treatment time.
2. RA was added to CG4 cells at two different concentrations (10 nM and 1 μM) at two distinct time points (4h or 1d). The cultures were subsequently grown in differentiation medium for a total of four days after which they were processed via immunocytochemistry for A2B5, O4, GalC, and MOG. The data collected regarding the individual markers were subjected to a two-way ANOVA, as well as a Bonferroni's multiple comparison post-test.
3. Statistical analysis revealed that significantly more $GalC^+$ cells are found in cultures treated with 1 μM RA at 1d than those treated with either 10nM RA at 1d or 1 μM RA at 4h.
4. Examination of the expression of PSA-NCAM, a potential downstream target of RA in oligodendroglial cells, revealed that significantly fewer PSA-$NCAM^+$ cells are found in cultures treated with either 1 μM RA at 4h or 10 nM RA at 1d than those treated with 10 nM RA at 4h.
5. The results of this experiment suggest that both RA concentration and treatment time can impact RA's effect on OG development.

6.2 Introduction

Oligodendroglial cells *in vivo* arise in multiple distinct foci (Cai et al. 2005; Kessaris et al. 2006; Vallstedt et al. 2005) and, therefore, must proliferate and migrate in order to populate specific CNS regions. In addition, these oligodendroglial cells must progress through four stages of development — OPCs, pro-OGs, pre-myelinating OGs, and mature myelinating OGs — [Figure 2.4] in order to myelinate axons in these regions. As pre-myelinating OGs do not actively proliferate (Fok-Seang and Miller 1994) or migrate (Noble et al. 1988), the terminal differentiation of these cells has to be delayed until they reach their final destination. Indeed, numerous TFs and signaling pathways

inhibit OG differentiation (Kondo and Raff 2000a; Kondo and Raff 2000b; Laeng et al. 1994; Noll and Miller 1994; Wang et al. 2001; Wang et al. 1998). Two of these signaling pathways (Notch and RA) also promote (Barres et al. 1994; Cui et al. 2004; Givogri et al. 2001; Hu et al. 2003; Pombo et al. 1999) this process. How these signaling pathways inhibit, as well as promote OG differentiation is uncertain, however, in the case of Notch it appears to be ligand-dependent. Activation of the Notch signaling pathway in purified OPCs by the standard Notch ligands, Jagged1 or Delta1, inhibits OG differentiation (Wang et al. 1998). In contrast, treatment of OPCs with F3 or NB-3, which represent a novel group of Notch ligands, promotes OG differentiation (Cui et al. 2004; Hu et al. 2003).

Although the reason for the differential effects of RA on OG differentiation is currently unknown, analysis of the experimental protocols utilized by different research groups [Table 6.1] suggests that it could be due to a stage- and/or concentration-dependent effect(s). Researchers have found that RA treatment of spinal cord cultures, which were obtained from either E14, E16, or E18 rats, inhibits OG differentiation with the effect being most dramatic in cultures obtained from the E14 rats (Noll and Miller 1994). As $O4^+$ pro-OGs and $GalC^+$ pre-myelinating OGs are known to first arise in the spinal cord at E16 and E18, respectively (Fok-Seang and Miller 1994), it could be hypothesized that RA inhibits OG differentiation at the $A2B5^+$ OPC stage. This, in turn, may explain why Laeng et al. (1994) and Givogri et al. (2001) report contradictory research findings. Although both research groups utilized cultures obtained from the cerebral hemispheres of neonatal rats, Laeng et al (1994) purified the cultures for OPCs prior to RA treatment whereas Givogri et al. (2001) did not. As a result, the cells utilized by Laeng et al (1994) would be expected to be more immature than those used by the other research group. However, as these two groups also utilized different concentrations of RA, the contradictory findings could also reflect a concentration-dependent effect. In order to determine which factors could impact RA's effect on OG differentiation we treated the oligodendroglial cell line CG4 with two different concentrations of RA at two distinct time points. The results show that both concentration and time can impact RA's effect on the differentiation of CG4 cells.

Table 6.1 Comparison of studies analyzing the effect of retinoic acid on oligodendrocyte differentiation

Study	Age	Source of OGs	[RA]	Differentiation
Barres et al. (1994)	P8	Optic nerve*	10nM all-*trans*/9-*cis*	+
Givogri et al. (2001)	P0-3 (7DIV)	Cerebral Hemispheres	1μM all-*trans*	+
Laeng et al. (1994)	P0	Cerebral Hemispheres*	8μM all-*trans*	-
Noll and Miller (1994)	E14, E16, E18	Spinal cord	1μM all-*trans*	-
Pombo et al. (1999)	P7	Optic nerve	30 or 500nM 9-*cis*	+

Abbreviations: *: purified for OPCs; +: enhance; DIV: days *in vitro*; -: inhibit

6.3 Materials and Methods

6.3.1 CG4 cells

The cells were first expanded in growth medium containing DMEM supplemented with 50 μg/ml apo-transferrin, 10 pg/ml D-biotin, 50 ng/ml sodium selenite, 5 μg/ml insulin, 1% antibiotic/antimycotic and 30% B104 conditioned medium. The cells were subcultured by rinsing them first with PUCKs [Section 5.3.3, pg. 82] and then incubating them for approximately 20 min in harvesting medium [EDTA and PUCKs (1:1) supplemented with 25 mM glucose, 2 mM sodium pyruvate, 10 mM HEPES, 1.25 μg/ml insulin, and 1% antibiotic/antimycotic]. Subsequently, the cells were collected and centrifuged at 800 rpm for 10 min, after which they were resuspended and plated in recovery medium [DMEM supplemented with 2 mM sodium pyruvate, 5% FBS, 5 μg/ml insulin, and 1% antibiotic/antimycotic]. At the start of the trial, the cells, which were suspended in recovery medium, were plated at a density of 100,000 cells/well onto poly-D-lysine-coated wells or coverslips in 24-well plates. Following cell attachment, the recovery medium was changed to a differentiation medium, which contained similar ingredients as the growth medium, except that it lacked B104 conditioned medium and, instead, contained 2% FBS. Subsequently, after either 4 h or one day, individual wells were either treated with 10 nM or 1 μM all-*trans* RA or left untreated for control purposes. (These RA concentrations were chosen based on previous studies [Table 6.1].) As RA is extremely light sensitive, cells were treated and maintained in the dark for this experiment. (Note: All of the chemicals were obtained from Sigma-Aldrich except for FBS, which was obtained from Invitrogen.)

6.3.2 Immunocytochemistry

Immunocytochemistry was conducted utilizing the following primary antibodies: A2B5 (1:100, hybridoma; ATCC), O4 (1:1000, Cedarlane Laboratories Ltd, Burlington, ON), anti-GalC (1:100, Ranscht et al., 1982), anti-myelin OG glycoprotein (MOG) (concentrate, kind gift of Dr. Minnetta Gardinier), anti-GFAP (1:800, Dako Canada Inc., Mississauga, ON), anti-PSA-NCAM (1:100, concentrate, Developmental Studies Hybridoma Bank), and anti-BrdU (1:400, Sigma-Aldrich, Oakville, ON). The CG4 cells were double stained for either A2B5/GalC or O4/MOG using an identical protocol. These markers were used to identify cells at different stages of oligodendrogenesis

[Figure 2.4]. The monoclonal antibody A2B5 was used to recognize a complex ganglioside expressed on the OPC's surface (Abney et al. 1983; Eisenbarth et al. 1979). The monoclonal antibody O4 recognized surface epitopes expressed by pro-OGs (Bansal et al. 1989). GalC, the major galactosphingolipid of myelin, marked pre-myelinating OGs (Ranscht et al. 1982). Finally, expression of myelin proteins, such as MOG, marked mature myelinating OGs. The CG4 cells were fixed with 4% formaldehyde, which was made from paraformaldehyde, for 10 min after which they were washed twice for 5 min each with PBS. This was followed by two 30-min incubations: first in the primary antibodies and then in the secondary antibodies [goat anti-mouse IgM μ-chain specific Alexa 594 (1:400, A2B5/O4; Invitrogen, Burlington, ON) and goat anti-mouse IgG γ-chain specific FITC (1:50, GalC/MOG; BIO/CAN Scientific, Mississauga, ON)]. In addition, the nuclei of all cells were stained by a 10-min incubation in Hoechst dye (Sigma-Aldrich, Oakville, ON). Two 5-min PBS washes followed each incubation. The cells/coverslips were mounted in ProLong® Gold antifade reagent (Invitrogen, Burlington, ON).

Double labeling of cultures for PSA-NCAM/GFAP was conducted as described above with minor alterations. For instance, the cells were first stained for PSA-NCAM and then GFAP. In addition, instead of PBS, the anti-GFAP antibody was diluted in 1% SM/0.03% TX. Finally, the secondary antibodies utilized were goat anti-mouse IgM μ-chain specific Alexa 594 (1:400, PSA-NCAM; Invitrogen, Burlington, ON) and goat anti-rabbit IgG Alexa 488 (1:200, GFAP; Invitrogen, Burlington, ON).

For staining proliferative cells, BrdU (Sigma-Aldrich, Oakville, ON) at a final concentration of 1 μM was added to the culture medium 1.5 h prior to processing. Subsequently, the CG4 cells were fixed for 10 min with 4% formaldehyde followed by two 5 min washes with PBS. The cells were then incubated at 37ºC for 30 min in 2N HCl. This was followed by two 5 min PBS washes and a 10 min block/permeablization in 3% SM/0.1% TX. The cells were then incubated for 1 h, 30 min, and 20 min in primary antibody, secondary antibody (goat anti-mouse IgG γ-chain specific FITC [1:50, BIO/CAN, Mississauga, ON]), and Hoechst counterstain, respectively. Two 5 min PBS washes were completed after each of these incubations. The cells/coverslips were then mounted in ProLong® Gold antifade reagent.

The percentage of cells that were stained with A2B5, O4, GalC, PSA-NCAM or BrdU in each treatment group was calculated by counting the number of immunoreactive and Hoechst^{+} cells on micrographs taken from every third field at 20 X magnification for a total of 25 fields utilizing the ImagePro® Express 5.1 software (Olympus Canada Inc., Markham, ON). Subsequently, the mean percentage of A2B5-, O4-, GalC-, or PSA-NCAM-immunoreactive cells in the control group was determined from three coverslips. As there was only one untreated control group for the four RA treatment groups the data were calculated as percentage of the control. In particular, the percentage of A2B5-, O4-, GalC-, or PSA-NCAM-immunoreactive cells from each individual RA-treated coverslip was divided by the mean percentage of the control cultures and multiplied by 100 in order to get a value which represented the percentage of the control. The mean value of three identically treated coverslips was then determined. These data were subsequently subjected to statistical analysis (two-way ANOVA and *post hoc* analysis conducted using Bonferroni's). For the BrdU incorporation experiment there were only 3 experimental groups. Therefore, the RA treatment groups were not calculated as a percentage of the control. Instead, the mean percentage of three coverslips from each experimental group was calculated in this case. As a result, the statistical analysis utilized a one-way ANOVA.

6.3.3 Reverse transcriptase-polymerase chain reaction

Samples were collected for RNA isolation by aspirating the medium and lysing the cells with 350 µl of Buffer RLT. The lysed cells were then collected in Eppendorf tubes, vortexed for 1 min, and stored at -80ºC until they were processed. The total RNA was extracted using the RNeasy® Mini kit (Qiagen Inc., Mississauga, ON) according to the manufacturer's instructions. cDNA was subsequently obtained using SuperScript™ Reverse Transcriptase (Invitrogen, Burlington, ON) as per the manufacturer's instructions except for the following modification: after the addition and gentle mixing of SuperScript™ Reverse Transcriptase the tubes were incubated at 25ºC for 10 min, 37ºC for 1.5 h, and finally 70ºC for 15 min. The EconoTAQ™ DNA Polymerase (VWR, West Chester, PA) kit was then used for PCR analysis. The following chemicals were added to the PCR tube: 2 µl cDNA, 5 µl 10X Buffer, 1.5 µl primer 1 and 2 (10 µM), 1 µl of each dNTP, 1 µl TAQ polymerase, sterile distilled water to 50 µl. The oligonucleotide DNA

primers utilized included:

Gene	Forward Primer	Reverse Primer
β-actin[a]	5′-ATTGTAACCAACTGGGACG-3′	5′-TTGCCGATAGTGATGACCT-3′
MBP[b]	5′-CTATAAATCGGCTCACAAGG-3′	5′-AGGCGGTTATATTAAGAAGC-3′
PST[c]	5′-ACTGAGGAGCACCAAGAGACGC-3′	5′-CCATGAAGGCAGGAATCCAAAGG-3′

a Hu et al (2004)
b www.hcnr.med.harvard.edu/programs/atrc/reagent_details/MBP%20Rat.pdf
c Soares et al. (2000); PST-Polysialyltransferase

PCR was performed in a PTC-100™ thermal cycler. After an initial denaturation step for 1.5 min at 94°C, 30 cycles were carried out with each cycle consisting of a 45 s denaturation step at 94°C, a 1 min annealing step at 55°C and a 1 min elongation step at 72°C. This was followed by a further elongation step for 4 min at 72°C. PCR products were analyzed by electrophoresis on 1% agarose gels using 1xTAE as the running buffer. The resulting bands, visualized with ethidium bromide, were photographed with the AlphaImager™ Gel Imaging System.

6.4 Results

6.4.1 Retionic acid's effect on the differentiation of CG4 cells is impacted by concentration and treatment time

RA was added to CG4 cells at two different concentrations (10 nM and 1 μM) at two distinct time points (4h and 1d) and left for a total of four days in culture. At this time, the cells were processed via immunocytochemistry for the OG markers A2B5, O4, GalC and MOG. As there was only a single untreated control group for this experiment the data were calculated as a percentage of control. Statistical analysis was not conducted on the MOG data since the number of MOG^+ cells was found to be minimal in all experimental groups at four days [Appendix B]. The remaining data sets were subjected to a two-way ANOVA with each OG marker being analyzed separately for statistical significance. Multiple *post hoc* comparisons were then performed utilizing Bonferroni's multiple comparison post-test. The two-way ANOVA revealed a significant main effect of concentration [$F(1,8)=23.93$, $p=0.0012$] but not time [$F(1,8)=0.040$, $p>0.05$] or interaction [$F(1,8)=0.829$, $p>0.05$] on the percentage (treatment/control) of $A2B5^+$ cells. In contrast, it failed to reveal any significant main effect on concentration [$F(1,8)=5.084$,

p>0.05], time [F(1,8)=0.721, p>0.05] or interaction [F(1,8)=1.174, p>0.05] in regards to the percentage (treatment/control) of $O4^+$ cells. The two-way ANOVA did, however, reveal a significant main effect of concentration [F(1,8)=9.444, p=0.0153] and time [F(1,8)=13.46, p=0.0063] but not interaction [F(1,8)=4.084, p>0.05] for GalC. Subsequently, Bonferroni's *post hoc* tests revealed that the percentage (treatment/control) of $A2B5^+$ cells is significantly lower in the 1 μM RA treatment groups in comparison to the 10 nM RA treatment groups regardless of the treatment time [Figure 6.1A]. It also revealed that the percentage (treatment/control) of $GalC^+$ cells is significantly higher in cells treated with 1 μM RA at 1d than those treated with either 1 μM RA at 4h or 10 nM RA at 1d [Figure 6.1C].

6.4.2 The mRNA expression of *MBP* is similar in cultures at four days regardless of the experimental group.

As all experimental groups contain only a few MOG-immunoreactive cells at four DIV, it was decided to examine the transcript expression of another myelin protein gene *MBP* utilizing RT-PCR. The results of this analysis suggest that *MBP* expression is similar in cultures at four days regardless of the experimental group [Figure 6.2].

6.4.3 The percentage of $BrdU^+$ cells present in cultures at one day is not affected by retinoic acid treatment.

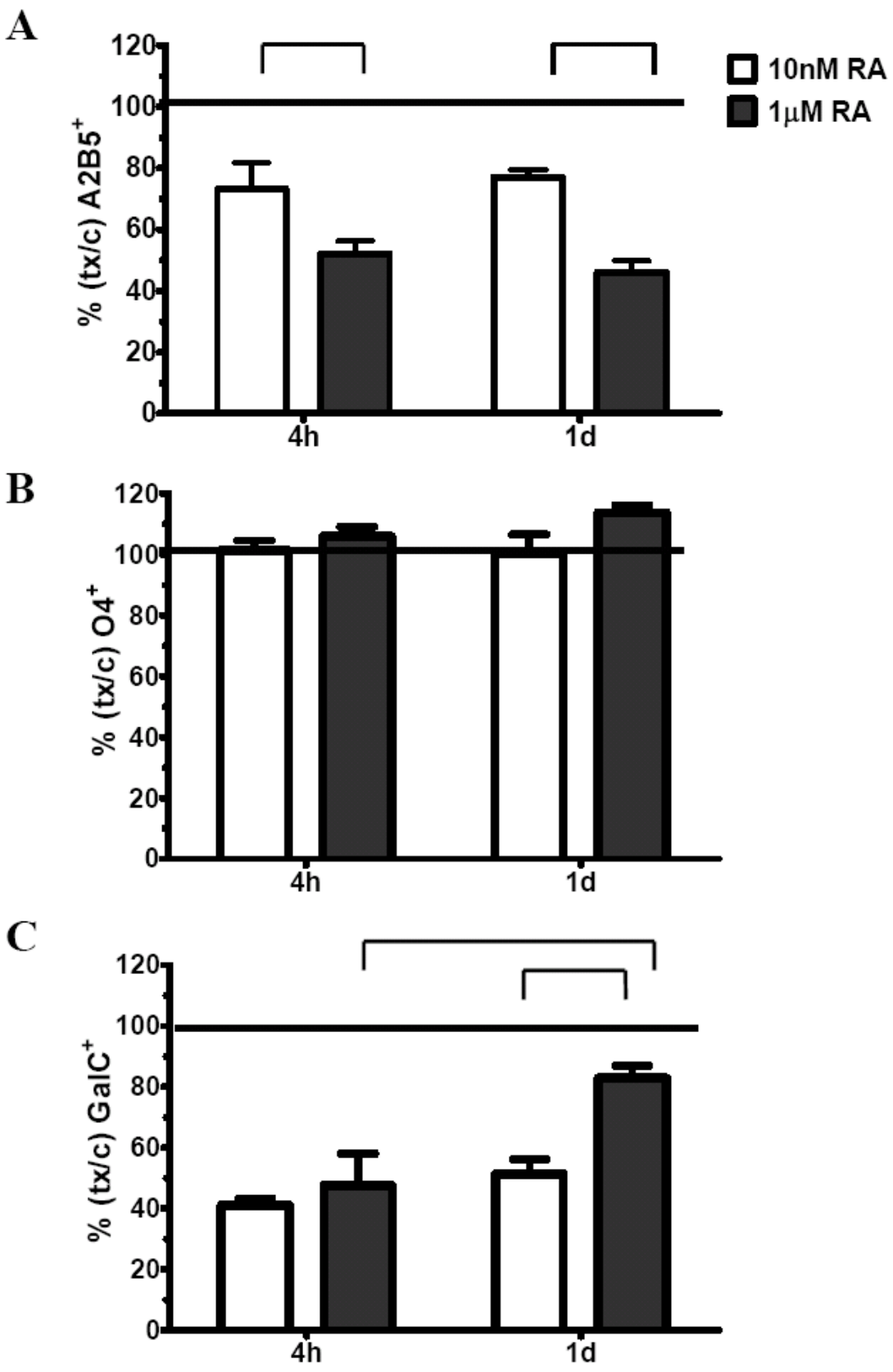
In order to determine if the concentration of RA could affect the proliferation of the CG4 cells, immunocytochemical analysis for BrdU was conducted at 1 DIV on control CG4 cells, as well as those treated with 10 nM or 1 μM RA at 4h. The data were subjected to a one-way ANOVA, which failed to reveal a significant main effect of concentration [F(2,4)=1.6, p>0.05] [Figure 6.3].

6.4.4 There are significantly more PSA-$NCAM^+$ cells in cultures treated with 10nM retinoic acid at 4h than those treated with either 10 nM at 1d or 1 μM retinoic acid at 4h.

A potential downstream target of RA signaling in oligodendroglial cells is PSA-NCAM. Therefore, PSA-NCAM expression was examined in each of the experimental groups after 4 DIV utilizing immunocytochemistry. The data obtained from this experiment were subjected to a two-way ANOVA, as well as Bonferroni's post-test. The

Figure 6.1 Retinoic acid's effect on CG4 cell differentiation is impacted by time and concentration.

CG4 cells, which were either left untreated or treated with 10 nM or 1 μM all-*trans* RA at 4h and 1d in culture, were grown *in vitro* for a total of four days. At this time three coverslips from each experimental group were stained for A2B5, O4 and GalC as described in Materials and Methods. The percentage [treatment group (tx) /control (c)] of A2B5-, O4-, and GalC-immunoreactive cells was then calculated for each RA treatment group. The black lines depict the control cultures, which were given a value of 100%. The histogram bars represent the mean of the percentages obtained from three individual coverslips, which were treated identically. Error bars represent standard error of the mean. *Post hoc* comparisons were performed with Bonferroni's multiple comparison post-test. This post-test revealed that the percentage of $A2B5^+$ cells is significantly lower ($p<0.05$; brackets) when treated with 1 μM RA versus 10 nM RA regardless of the time it was added (A). In comparison, the percentage of $O4^+$ cells is found to be similar regardless of time and RA concentration (B). The percentage of $GalC^+$ cells is significantly lower ($p<0.05$; brackets) in the 1 μM 4h and 10 nM 1d treatment groups than the 1 μM 1d group (C).


A
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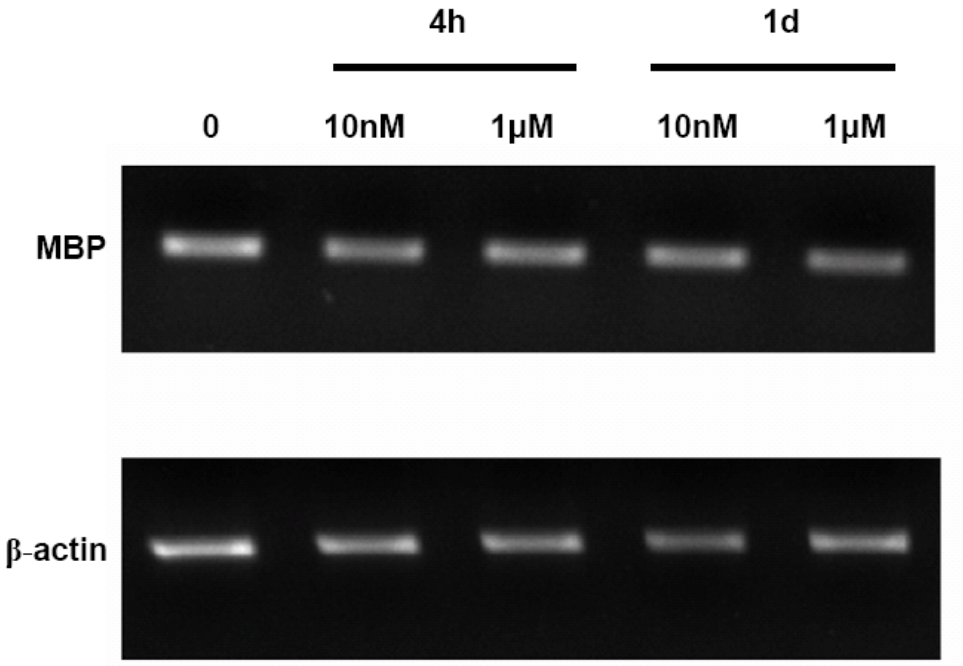

Figure 6.2 The expression of *MBP* in CG4 cells treated with retinoic acid.

CG4 cells, which were either left untreated or treated with 10 nM or 1 μM all-*trans* RA at 4h and 1d in culture, were grown *in vitro* for a total of four days. At this time the cells were collected and processed for RT-PCR for the analysis of *MBP* and *β-actin* gene expression. Given equal amounts of cDNA, as shown with *β-actin*, the expression level of *MBP* did not appear to vary in the different experimental groups.

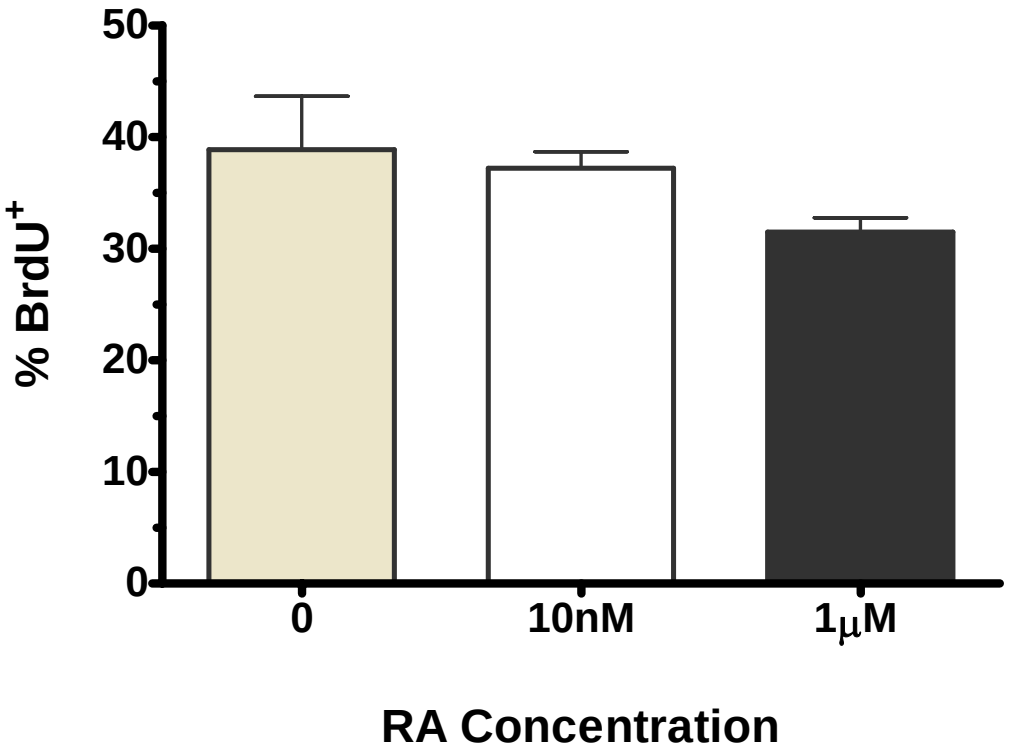

Figure 6.3 The percentage of BrdU$^+$ cells is not significantly affected by retinoic acid treatment.

CG4 cells, which were either left untreated or treated with 10 nM or 1 μM all-*trans* RA at 4h and 1d in culture, were grown *in vitro* for a total of four days. At this time three coverslips from each experimental group were stained for BrdU as described in Materials and Methods. The histogram bars represent the mean of the percentages obtained from three individual coverslips, which were treated identically. The error bars represent the standard error of the mean. The data were analyzed utilizing a one-way ANOVA test and found to be not significant [$F(2,4)=2$, $p>0.05$].

two-way ANOVA revealed a significant main effect of concentration [$F(1,8)=55.56$, $p<0.0001$] and interaction [$F(1,8)=19.86$, $p=0.0021$] but not treatment time [$F(1,8)=1.641$, $p>0.05$]. Subsequently, the *post hoc* test revealed that the percentage (treatment/control) of PSA-NCAM$^+$ is significantly higher in cells treated with 10 nM RA at 4h than those treated with either 1 μM RA at 4h or 10 nM RA at 1d [Figure 6.4]. As PSA-NCAM is a post-translational modification of the NCAM molecule, RT-PCR analysis was also conducted to determine whether the expression of *PST*, which is the enzyme responsible for adding polysialic acid units to the NCAM molecule in oligodendroglial cells (Stoykova et al. 2001), is affected by RA treatment. The results of this analysis show that *PST* expression only appears to be reduced in the cells treated with 1 μM RA regardless of the time [Figure 6.5].

6.5 Discussion

CG4 cells (Louis et al. 1992) were utilized instead of primary cultures to study the effect of RA on OG development because they exhibit several advantages over primary cultures, including accessibility and increased rate of differentiation. These cells not only display numerous characteristics of normal oligodendroglial cells including morphological and antigenic phenotypes, but they also respond similarly to mitogens (Louis et al. 1992).

In this study CG4 cells were treated with two concentrations of RA (10 nM and 1 μM) at either 4h or 1d and then left for a total of 4 DIV. RA's effect on OG differentiation was then determined by conducting immunocytochemical analysis for the OG markers A2B5, O4, GalC, and MOG. The results suggest that RA decreases A2B5 expression in a concentration-dependent manner [Figure 6.1A]. Although this result is unexpected, there are several possible explanations that may account for this reduction. For instance, previous research has suggested that RA inhibits the progression of OPCs to pro-OGs (Noll and Miller 1994). Although A2B5 is often utilized to identify OPCs, it also labels pro-OGs (Fok-Seang and Miller 1994). Therefore, this reduction could reflect a decrease in the percentage of A2B5$^+$O4$^+$ CG4 cells with RA treatment. In turn, a decrease in the percentage of A2B5,O4-double immunoreactive cells would indicate that the CG4 cells are not progressing through to later stages of development. However, as

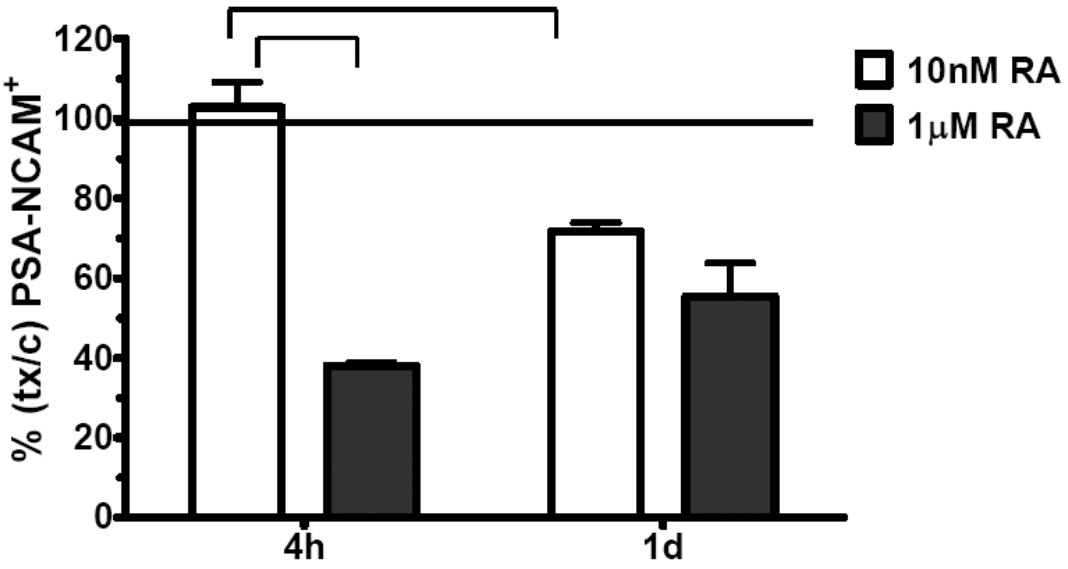

Figure 6.4 The percentage of PSA-NCAM$^+$ cells is significantly lower in cells treated with 10 nM retinoic acid at 1d and 1 μM retinoic acid 4h than in cells treated with 10 nM retinoic acid at 4h.

CG4 cells, which were either left untreated or treated with 10 nM or 1 μM all-*trans* RA at 4h and 1d in culture, were grown *in vitro* for a total of four days. At this time three coverslips from each experimental group were stained with an anti-PSA-NCAM monoclonal antibody as described in Materials and Methods. The percentage [treatment group (tx)/control (c)] of PSA-NCAM-immunoreactive cells was calculated for each RA treatment group. The black line depicts the control culture, which was given a value of 100%. The histogram bars represent the mean of the percentages obtained from three individual coverslips, which were treated identically. Error bars represent the standard error of the mean. *Post hoc* comparisons were performed with Bonferroni's multiple comparison post-test. This post-test revealed that the percentage of immunoreactive cells is significantly lower ($p<0.05$; square brackets) in the 10 nM 1d and 1 μM 4h treatment groups compared to the 10 nM 4h group.

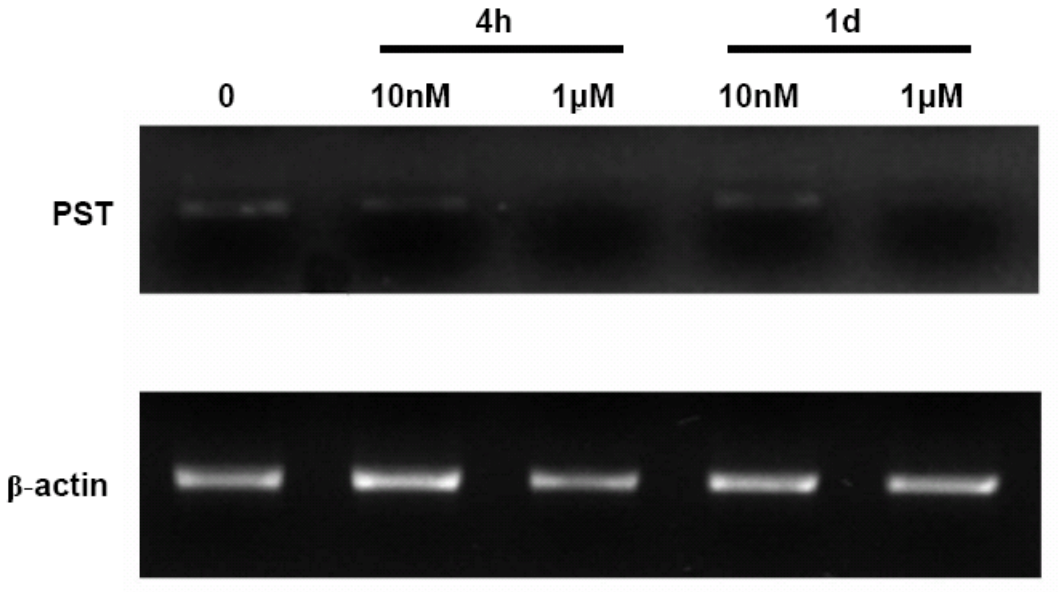

Figure 6.5 The expression of *PST* appears to be reduced in CG4 cells treated with 1μM retinoic acid.

CG4 cells, which were either left untreated or treated with 10 nM or 1 μM all-*trans* RA at 4h and 1d in culture, were grown *in vitro* for a total of four days. At this time the cells were collected and processed via RT-PCR for *PST* and *β-actin*. The results of this experiment, which was performed in triplicate, suggests that treatment of cells with 1 μM RA at either 4h or 1d reduces the expression of *PST* compared to cells treated with either 10 nM RA or those left untreated.

these monoclonal antibodies are of the same species and class, we were unable to address this issue directly. However, analysis of the percentage (treatment/control) of $O4^+$ cells suggests that this is unlikely since it is similar regardless of the concentration utilized [Figure 6.1B].

Besides oligodendroglial cells, the A2B5 antibody also recognizes type 2 astrocytes. As CG4 cells can develop into either type 2 astrocytes or OGs (Louis et al. 1992), the reduction in the percentage of $A2B5^+$ cells could reflect a negative effect of RA on the development of type 2 astrocytes. However, as the expression of GFAP was negligible in all cultures analyzed, this explanation is unlikely.

As RA has been shown to inhibit proliferation in a concentration-dependent manner (Laeng et al. 1994), the reduction could reflect a decrease in the number of proliferative cells. In order to address this possibility the cells were treated for one and a half hours with BrdU after one day in culture. Cells were then processed and the percentage of BrdU-immunoreactive cells was determined. Although the results of this experiment failed to reveal a significant difference between the RA-treated and control groups [Figure 6.2], the cells were not double-labeled with oligodendroglial markers and, hence, we are unable to comment on whether the proliferation at a particular stage of development was affected by RA. Furthermore, we cannot address whether the proliferation of the cells changed with more time in culture.

RA's effect on the percentage (treatment/control) of $A2B5^+$ cells could also be due to a concentration-dependent effect on apoptosis. Recent research has shown that RA can induce apoptosis in cells containing high concentrations of cellular RA-binding protein II (CRABPII), which shuttles RA to the RARs responsible for promoting apoptosis (Schug et al. 2007). How it accomplishes this is unknown, but it may involve its ability to induce the expression of certain proapoptotic genes, including *caspase 7* and *caspase 9* (Donato and Noy 2005). Although the total number of cells in the different experimental groups was found to be similar in our study, all cultures contained fragmented nuclei indicative of dead cells. Further research will be needed to determine if RA enhances apoptosis in oligodendroglial cells.

Analysis of the percentage of $GalC^+$ cells revealed a time-dependent effect with respect to the 1 μM RA treatment group. In particular, cultures treated with 1 μM RA at

either four hours or one day are found to contain 47.6% and 82.9% GalC$^+$ cells, respectively, of the control cultures [Figure 6.1C]. Hence, this suggests that 1 μM RA is less effective at impairing CG4 cell differentiation when it is added after one day instead of four hours. In comparison, treatment of CG4 cells with 10 nM RA resulted in a similar reduction in the percentage of GalC$^+$ cells regardless of the time it was added. One possible explanation for this time/concentration-dependent effect is that higher concentrations of RA at later stages of development may regulate additional effector genes that actually promote the differentiation of CG4 cells. For example, RA has been shown to enhance *MBP* expression (Givogri et al. 2001). Therefore, in order to see if RA's effect on *MBP* mRNA expression could be impacted by concentration and/or treatment time, RT-PCR analysis was conducted. Although the results imply that *MBP* expression is similar regardless of the experimental manipulation [Figure 6.3], subtle changes may have been missed due to the non-quantitative nature of our RT-PCR analysis. Further research will be needed to determine the cause of the concentration/time-dependent effect.

How RA can impair CG4 cell differentiation is unknown; however, it may involve its ability to augment the expression of PSA-NCAM (Husmann et al. 1989), which has been implicated in inhibiting OG differentiation (Decker et al. 2000). However, contrary to our hypothesis we find that the percentage of PSA-NCAM$^+$ cells is significantly higher in cells treated with 10 nM RA at 4h than those treated with either 1 μM RA at 4h or 10 nM RA at 1d [Figure 6.4]. This was unexpected based on the findings of Decker et al. (2000), who report an increase in the percentage O4$^+$ cells following endoN treatment. Closer examination of their data, however, reveals that this effect is only evident under certain *in vitro* conditions (Decker et al. 2000). Furthermore, a similar experiment conducted by a different research group failed to reveal any differences in the percentage of GalC$^+$ cells in endoN-treated and control cultures (Zhang et al. 2004). Based on this information, as well as our results, it appears that RA's effect on CG4 cell differentiation is independent of its effect on PSA-NCAM expression. Although RA's effect on PSA-NCAM expression is opposite of what was expected, it may be due to the fact that Hussman et al. (1989) utilized a carcinoma cell line whereas we used CG4 cells.

Although RA's effect on PSA-NCAM cannot account for its effect on differentiation, it suggests that it could also impact oligodendroglial cell migration. This is due to the fact that PSA-NCAM is important in PDGF-directed OPC migration (Zhang et al. 2004). In order to examine possible mechanisms by which RA could reduce PSA-NCAM expression, the mRNA levels of *PST*, which is the main enzyme responsible for adding polysialic acid units to the NCAM molecule in oligodendroglial cells (Stoykova et al. 2001), was determined via RT-PCR. In comparison to control cultures *PST* expression appeared to be reduced in cells treated with 1 μM RA but not 10 nM RA [Figure 6.5]. Hence, these results suggest that RA, at least at higher concentrations, may repress PSA-NCAM expression in CG4 cells, in part, by repressing *PST* expression.

In conclusion, the results of this experiment highlight the complexity of RA's effects on oligodendroglial cells. In addition, they suggest that further research is needed to identify downstream effectors and targets of the RA signaling pathway active at various stages of OG development.

7.0 GENERAL DISCUSSION

Hoxa2 is expressed in multiple CNS regions (Hao et al. 1999; Tan et al. 1992; Wolf et al. 2001) and cell types (Hao et al. 1999; Nicolay et al. 2004a). However, our current understanding of its role in CNS development is limited to early stages of development, such as anterior-posterior (Gavalas et al. 1997) and dorsal-ventral (Davenne et al. 1999) patterning of the hindbrain, as well as neurogenesis (Gaufo et al. 2004). Although it is expressed by astrocytes and OGs (Hao et al. 1999), its role in the development of these cells is currently unknown. Therefore, the main purpose of this thesis was to investigate the expression and function of Hoxa2 during oligodendrogenesis. The research conducted is the first to show that Hoxa2 is expressed throughout OG development, diminishing only with the acquisition of a mature myelinating phenotype (Nicolay et al. 2004a) [Figure 3.3]. However, as this data was obtained prior to the identification of dorsal and ventral OPCs, further research is needed to determine if Hoxa2 expression is lineage specific. In turn, this may explain why Hoxa2 expression is limited to a small subset of PDGFαR-immunoreactive cells in the telencephalon [Figure 5.3].

Although Hoxa2 is expressed by A2B5$^+$ OPCs [Figure 3.3] it does not appear to be required for OG specification in the pMN domain [Figure 3.5] or the telencephalon [Figure 5.2]. It should be kept in mind, however, that its role in these regions may have been overlooked due to the fact that we did not quantify the immunoreactive cells in the presence and absence of *Hoxa2*. Furthermore, with the recent identification of ventral and dorsal OPCs arising from multiple CNS regions further research will be needed to determine whether Hoxa2 could be important for OG specification in specific CNS regions and/or OPC lineages. In fact, circumstantial evidence suggests a role for Hoxa2 in OG specification in the embryonic hindbrain. In particular, researchers have found that Lbx1-expressing somatic sensory interneuron precursors, which arise from dp4-6 in the neural tube (Gross et al. 2002), are lost in r2 and dramatically reduced in r3 in *Hoxa2* mutant mice (Gaufo et al. 2004). As dorsal OPCs appear to arise from dp3-5 in the neural tube (Cai et al. 2005), Hoxa2 may affect OG specification in this region. In addition, expression of *Pax3*, which overlaps with that of *Olig1* in the dorsal hindbrain

(Vallstedt et al. 2005), is expanded ventrally in r2 and r3 of *Hoxa2* mutant mice (Davenne et al. 1999).

At these rhombomeric levels OPCs also arise from the pMNv domain, which is defined by the TF Nkx2.2 (Vallstedt et al. 2005). Although Davenne et al. (1999) reported that *Nkx2.2*'s expression profile displays a normal distribution in the hindbrain of *Hoxa2*-/- mice, they found that these mice exhibit a down-regulation of *Pax6* expression in r2 and r3. As Pax6 and Nkx2.2 cross-repress one another in the neural tube (Briscoe et al. 2000), it is somewhat surprising, therefore, that *Nkx2.2* expression was not altered in r2 and r3 of these mutant mice. One drawback of this study, however, was the fact that the researchers examined expression profiles utilizing dorsal views of flat-mounted hindbrains (Davenne et al. 1999). This, in turn, could have made it difficult to identify subtle changes in expression profiles along the dorsal-ventral axis. Support for this statement comes from the finding that *Phox2b* expression shows a dorsal expansion of its r2 ventral expression column in *Hoxa2*-/- versus WT mice (Davenne et al. 1999). Phox2b$^+$ MNs arise from the Nkx2.2$^+$ pMNv domain (Pattyn et al. 2003). Furthermore, *in ovo* electroporation of chick embryos with a *Nxk2.2*-expressing vector results in ectopic expression of *Phox2b* (Pattyn et al. 2003). Hence, this information suggests that there may be a change in *Nkx2.2*'s expression profile that has been overlooked. Further research is needed in order to resolve this issue, as well as to examine Hoxa2's role in OG specification in r2 and r3.

In addition to Hoxa2, circumstantial evidence suggests that other Hox TFs could be important for OG specification in specific rhombomeres. For example, Olig2 expression, which specifically demarcates the pMN domain (Gaufo et al. 2003; Novitch et al. 2001), is absent in r5 in *Hoxa3*-/-;*Hoxb3*-/- mice (Gaufo et al. 2003). Accordingly, these mice exhibit a loss of somatic MNs in this region (Gaufo et al. 2003). As somatic MNs arise from the pMN domain prior to OGs (Fu et al. 2002), these results suggest that Hoxa3 and Hoxb3 could be important for OG specification in r5. Interestingly, Olig2 expression in the pMN domain is also dramatically decreased in *Raldh2* mutant mice (Novitch et al. 2003). Therefore, this suggests that Hox TFs could affect OG specification as downstream effectors in the RA signaling pathway. Further research is needed to verify this. In addition, further research is needed to examine the role of

Hoxa2 in different OPC lineages. In order to determine its function(s) in a specific lineage, however, the elimination of the other OPC lineages may be required.

In comparison to specification, the research conducted utilizing the CG4 cell line has demonstrated for the first time a role for Hoxa2 in OG differentiation [Figure 5.4]. Although the mechanism by which it affects differentiation is unknown, we speculated that it may be mediated by PSA-NCAM. Contrary to our hypothesis, however, we found that the percentage of PSA-NCAM$^+$ cells is significantly lower in cells over-expressing *Hoxa2* [Figure 5.5]. Therefore, it appears unlikely that Hoxa2's effect on OG differentiation is mediated by PSA-NCAM.

Due to the fact that *Hox* genes are regulated by RA (Simeone et al. 1990), we also examined the effect of RA treatment on CG4 cells. The results of this experiment showed that the effect of RA on CG4 cell differentiation is affected by its concentration, as well as the treatment time [Figure 6.1]. Furthermore, the percentage of PSA-NCAM$^+$ cells is significantly lower in the 1 μM RA 4h and 10 nM RA 1d treatment groups than the 10 nM RA 4h treatment group [Figure 6.4]. Although it was not directly demonstrated, the results suggest that RA's effect on OG differentiation and PSA-NCAM expression may be mediated in part by Hoxa2.

How RA could inhibit OG differentiation is unknown but it may involve its ability to enhance *Sox9* and *Sox6* expression. In particular, RA enhances the mRNA expression of both *Sox9* (Sekiya et al. 2000) and *Sox6* (Hamada-Kanazawa et al. 2004a; Hamada-Kanazawa et al. 2004b) in specific cell lines. In addition, researchers have identified three potential RAREs upstream of the *Sox9* promoter (Afonja et al. 2002). As Sox9 enhances *Sox6* mRNA expression (Stolt et al. 2006), RA-induced expression of *Sox9* could indirectly enhance the expression of this SoxD TF. Sox6 could then inhibit OG differentiation via interfering with the function of Sox10. Interestingly, there is circumstantial evidence to suggest that Hox TFs could be involved in this signaling pathway since potential HBSs have been identified in putative regulatory elements of the *Sox9* gene (Akiyama et al. 2007), therefore, suggesting that *Sox9* could be a downstream target of Hox TFs.

As RA also promotes OG differentiation (Barres et al. 1994; Pombo et al. 1999), the most obvious question that arises is whether Hox TFs could also act downstream of

RA in this process. There are 39 mouse and human *Hox/HOX* genes (Akin and Nazarali 2005; Favier and Dollé 1997; Santagati and Rijli 2003; Scott 1992); therefore, specific Hox TFs could either inhibit or promote OG differentiation. Alternatively, evidence suggests that the role of Hox TFs, including Hoxa2, could be dependent on its surrounding environment. Researchers have found that certain Smads (for Sma and Mad), which are downstream effectors in the BMP signaling pathway (Kloos et al. 2002), can modulate the DNA binding and transcriptional activities of Hox TFs (Li et al. 2006). Resolution of this issue will have to await further research.

Although RA's and Hoxa2's effect on PSA-NCAM expression cannot account for their effect on OG differentiation, it suggests that Hoxa2 and RA could have additional roles in OG development. In particular, PSA-NCAM is important for PDGF-directed OPC migration (Zhang et al. 2004). Hence, based on the above results it could be hypothesized that RA and Hoxa2 could negatively impact OPC migration.

PSA-NCAM is also expressed by neurons; however, its expression decreases with a time frame that corresponds with the onset of myelination (Charles et al. 2000). Researchers have found that removing the polysialic acid moiety from the NCAM molecule results in an increase in the number of myelinated internodes (Charles et al. 2000). As Hoxa2 is expressed by neurons (Nicolay et al. 2004a)[Figure 3.4] the above findings, along with our data, would suggest that over-expressing *Hoxa2* in neurons could lead to an increase in the number of myelinated axons. Interestingly, researchers have found that PSA-NCAM is re-expressed by demyelinated axons but not remyelinated axons in MS plaques (Charles et al. 2002). Therefore, this suggests that PSA-NCAM does not favor myelination in both normal, as well as disease states.

The findings described in this thesis not only represent the first step in the process of determining the function(s) of Hoxa2, but it also provides an indication of the role(s) of other Hox TFs in OG development. In turn, the ultimate goal for elucidating the regulation of oligodendrogenesis is to be able to utilize the information to develop new therapeutic treatments for MS. MS is an inflammatory disease of the CNS characterized by focal areas of demyelination that appear to be immune driven (Hemmer et al. 2006; Lucchinetti et al. 1999; Ozawa et al. 1994; Prineas et al. 1993a; Prineas et al. 1993b; Raine 1997a; Raine 1997b; Storch et al. 1998). The resulting plaques, which can vary in

diameter from less than a millimeter to several centimeters (Al-Omaishi et al. 1999), can occur anywhere within the white matter but favor the periventricular regions, optic nerves, brainstem, cerebellum and spinal cord (Wingerchuk et al. 2001). Early symptoms often include varying degrees of one or more of the following: weakness or reduced dexterity in one or more limbs, monocular vision loss, double vision, gait instability, and ataxia. As the disease worsens, the majority of patients exhibit bladder dysfunction, fatigue, and heat sensitivity (Hauser and Oksenberg 2006). Based on the frequency and duration of their symptoms MS patients can be divided into three groups. Approximately 85% of patients present with relapsing-remitting MS (RR-MS), which is characterized by acute attacks of worsening neurological function followed by a variable degree of recovery (Hauser and Oksenberg 2006; Lublin and Reingold 1996). In many cases RR-MS develops into progressive disability at which point the patients are diagnosed with secondary progressive MS. In contrast, around 15% of patients display a relatively steady decline in neurological function and are, therefore, said to have primary progressive MS (PP-MS) (Hauser and Oksenberg 2006; Lublin and Reingold 1996). Interestingly, although the median age of onset of MS is approximately 28 (Wingerchuk et al. 2001), patients presenting with PP-MS are usually older than those with RR-MS (Hauser and Oksenberg 2006).

Currently most therapeutic treatments for MS are aimed at managing individual symptoms (Crayton et al. 2004) or the inflammatory/immune response (Hauser and Oksenberg 2006). However, there is increasing attention being given to developing therapeutic treatments aimed at promoting remyelination of the demyelinated regions. Remyelination, which is characterized by axons exhibiting thin myelin sheaths, does occur to some extent in MS but fails with disease progression (Brück et al. 1994; Ozawa et al. 1994; Prineas et al. 1993a; Prineas et al. 1993b; Prineas et al. 1989; Raine and Wu 1993). Although there are two possible sources of remyelinating cells, adult OPCs or surviving OGs, current research supports the former (Chang et al. 2000; Gensert and Goldman 1997; Levine and Reynolds 1999). Hence, remyelination would require these adult OPCs to proliferate, migrate, differentiate, and finally remyelinate the demyelinated axons. Recent research has found that during remyelination the expression profiles of several TFs are reminiscent of those seen during normal development (Arnett et al. 2004;

Fancy et al. 2004; Sim et al. 2002; Watanabe et al. 2004). Hence, it could be postulated that these TFs play similar roles in both instances. Although the function of most TFs has not been analyzed in the context of remyelination, there is evidence supporting this statement for Olig1. In particular, in the absence of *Olig1*, *PLP/DM20* and *MBP* expression is markedly reduced in the P0 (Lu et al. 2002) and P14 (Xin et al. 2005) spinal cord and undetectable in the P14 corpus callosum, neocortex and striatum (Xin et al. 2005). Although the density of *PLP/DM20*-expressing cells is found to be similar in the adult unlesioned spinal cord of WT and *Olig1*-/- mice, it is significantly decreased in the mutant mice two weeks following lysolecithin-induced demyelination (Arnett et al. 2004). Hence, these results suggest that Olig1 is important for OG differentiation during normal development, as well as during remyelination.

Knowledge regarding the role(s) of particular signaling pathways and TFs during normal oligodendrogenesis may be useful in identifying potential reasons for remyelination failure in MS. For instance, researchers have found that Notch1 and its downstream effector, Hes5, are expressed by immature oligodendroglial cells within and around active MS lesions lacking remyelination. The Notch ligand, Jagged1, is also expressed in these lesions but not in lesions exhibiting remyelination (John et al. 2002). As a result, it could be postulated that prolonged activation of the Notch signaling pathway by standard Notch ligands could inhibit OG differentiation and, hence, impair remyelination. Initial support for this statement comes from the recent finding that inhibition of Notch signaling in mice with experimental autoimmune encephalomyelitis is associated with increased remyelination and decreased axonal damage in comparison to control (Jurynczyk et al. 2005). Therefore, identifying transcriptional cascade anomalies within demyelinated lesions may lead to the development of therapeutic treatments aimed at enhancing endogenous remyelination.

Furthermore, knowledge regarding the transcriptional regulation of normal OG development may also aid in establishing stem cell therapy as a feasible means of remyelinating MS lesions. For instance, the success of this treatment depends upon the ability of directing these cells to become OGs. As the TF Olig2 is important for OG specification in various regions of the embryonic CNS (Lu et al. 2002), it may be useful in this regard. Indeed, researchers have found that transfecting NSCs obtained from

embryonic murine brains with *Olig2* results in most cells becoming mature OGs. Furthermore, in contrast to control NSCs, these *Olig2*-transfected NSCs are found to actively participate in remyelinating cuprizone-induced demyelinated regions in the corpus callosum following transplantation (Copray et al. 2006). Hence, understanding the transcriptional control of normal oligodendrogenesis will aid in the development of multiple therapeutic treatments aimed at promoting remyelination of MS lesions.

It becomes evident from the above discussion, however, that researchers need to be aware of the fact that a specific treatment may affect more than the cell of interest. For example, whereas removal of the polysialic acid moiety from the NCAM molecule in neurons would likely aide in remyelination, its removal from OPCs may hinder remyelination by impairing the migration of these cells. In addition, although information regarding the regulation of OG development will be extremely beneficial in determining mechanisms by which to promote remyelination in MS lesions, it has to be kept in mind that the demyelinating environment could potentially alter the function of particular TFs.

8.0 FUTURE DIRECTIONS

8.1 Hoxa2

Although the findings of this thesis primarily support a role for Hoxa2 in OG differentiation, there is reason to believe that it could also affect oligodendroglial specification, migration, and myelination. For instance, TF patterning defects evident in r2 and r3 of *Hoxa2*-/- mice (Davenne et al. 1999) suggest that OG specification could be affected in these regions of the hindbrain. In order to address this issue, immunohistochemical and/or *in situ* hybridization histochemical analysis should be conducted on transverse hindbrain sections of *Hoxa2* transgenic mice at the levels of r2 and r3 utilizing the following markers: Nkx2.2, Pax6, Olig2, Mash1, Ngn2, Pax3, Gsh1, and Dbx2. Each of these TFs has been selected for specific reasons. In particular, Nkx2.2 was chosen because it specifically demarcates the pMNv domain from which ventral OPCs arise in r2 and r3 (Pattyn et al. 2003). Furthermore, Nkx2.2 appears to be important for OG specification in the anterior hindbrain since a dorsal expansion of its expression domain, as seen in *Nkx6.1*;*Nkx6.2* double mutant mice, is associated with ectopic OG development (Vallstedt et al. 2005). In turn, Pax6 was selected since it defines the dorsal boundary of the pMNv domain along with Nkx2.2 (Pattyn et al. 2003). In addition, its mRNA expression is reduced in r2 and r3 of *Hoxa2-/-* mice (Davenne et al. 1999). Olig2 was chosen because it is expressed by OPCs in both ventral and dorsal regions of the hindbrain (Fu et al. 2002; Vallstedt et al. 2005). Similarly, Pax3 was selected since dorsal OPCs have been shown to arise within its neuroepithelial expression domain (Vallstedt et al. 2005). Furthermore, a ventral shift in *Pax3*'s expression profile has been found in r2 and r3 of *Hoxa2-/-* mice (Davenne et al. 1999). *Gsh1* and *Dbx2* were included because they define the ventral limit of the dorsal OPC domain (Vallstedt et al. 2005). Finally, *Mash1* and *Ngn2* were selected since both factors exhibit expression anomalies in r2 and r3 of *Hoxa2-/-* mice (Davenne et al. 1999). Furthermore, down-regulation of these TFs appears to be important for the switch from neurogenesis to gliogenesis (Tomita et al. 2000; Zhou et al. 2001). Due to the fact that the anomalies in gene patterning exhibited by *Hoxa2-/-* mice are seen at E10.5 (Davenne et al. 1999), histochemical analyses should first be conducted at this age in order to confirm these

findings, as well as look at other TFs. Subsequently, it should also be conducted at E13.5 since both dorsal and ventral OPCs are found in the hindbrain at this time (Vallstedt et al. 2005). Based on the initial findings it may be necessary to include an intermediary age, such as E12, in order to determine if there are any anomalies in the switch from neurogenesis to gliogenesis in these areas. This would, in turn, require the inclusion of the neuronal marker Islet1, as well as the oligodendroglial marker PDGFαR. Overall these experiments will enable the researcher to identify if there are any anomalies in temporal and/or spatial aspects of OG specification in both ventral and dorsal regions of r2 and r3.

In comparison to OG specification, potential roles for Hoxa2 in migration and myelination are based on the finding that *Hoxa2* over-expression results in significantly fewer PSA-NCAM^{+} cells. As PSA-NCAM is important for PDGF-directed migration, it suggests that Hoxa2 could negatively impact this trait. To test this directly, migration assays should be conducted utilizing WT and Hoxa2-S CG4 cells according to the protocols described in Zhang et al. (2004). In turn, as PSA-NCAM axonal expression appears to negatively impact myelination (Charles et al. 2000), it suggests that Hoxa2 could play a favorable role in this process. As a result, it would be interesting to see if PSA-NCAM axonal expression is up-regulated in r2 and r3 of *Hoxa2*-/- mice and whether this up-regulation could negatively impact myelination. However, as the *Hoxa2* mutant mice die shortly after birth, r2/r3 explant cultures would have to be used in order to see if there are any anomalies in myelination.

Further research is also needed in order to determine how Hoxa2 regulates PSA-NCAM expression. Although preliminary evidence suggests that it could involve multiple mechanisms, such as regulating NCAM and *PST* expression, it is currently unknown whether this regulation is direct or indirect. In regards to NCAM expression, evidence suggests that Hoxa2's effect could be direct since HBSs have been found in the NCAM promoter region (Hirsch et al. 1990). However, in order to test this directly, CG4 cells should be co-transfected with a *Hoxa2*-expressing plasmid, as well as a NCAM-luciferase reporter construct utilizing the protocol described in Nielsen et al. (2004). In comparison to NCAM, it is currently unknown whether HBSs are present in the promoter region of the *PST* gene. Therefore, the first step would be to examine the PST promoter

region for potential Hoxa2-binding sites (Zeynep Akin's PhD Thesis 2004). If potential binding sites are identified, electrophoretic mobility shift assays should be conducted in order to show that Hoxa2 can indeed bind to these sites. Luciferase assays should then be conducted if Hoxa2-binding sites are found in the promoter region.

In addition to normal OG development, I believe it would be worthwhile to see if Hoxa2 could affect the ability of oligodendroglial cells to actively participate in the remyelination. Previous research has shown that transplanted CG4 cells actively participate in repairing regions of persistent demyelination within the X-irradiated rat spinal cords (Franklin et al. 1996). Therefore, I would suggest repeating this experiment utilizing WT and Hoxa2-S CG4 cells in order to determine if there are any discrepancies in the remyelination capabilities of these two cultures.

Finally, research will need to be conducted in order to determine the mechanism by which Hoxa2 affects OG differentiation. I would suggest utilizing RT-PCR and/or western blot analysis in order to determine if the expression of Sox9 and/or Sox6 is enhanced in the Hoxa2-S CG4 cultures in comparison to the WT.

8.2 Retinoic acid

Although there is much research supporting a role for RA in OG development, little is known regarding RA signaling in oligodendroglial cells. For instance, information regarding the expression of Raldhs, CYPs, RARs, and RXRs during OG development is extremely limited. Therefore, I would suggest conducting double immunofluorescent analysis *in vitro* and *in vivo* in order to determine if the expression of these factors changes as oligodendroglial cells progress from precursors to mature myelinating cells. In turn, the information obtained from these studies could subsequently be utilized in the development of future experiments. For example, if Raldhs are found to be expressed by differentiated OGs but not OPCs, this would suggest that only the former is capable of synthesizing RA. This, in turn, could be subsequently tested in culture assays utilizing the protocol previously described by Mey and Hammelmann (2000). Similarly, if OPCs express different RARs than OGs, this would suggest that these receptors are active at different stages of OG development. This, then, could be tested in culture assays via activating specific RARs at different stages of OG

development utilizing retinoid specific agonists, such as those described in Goncalves et al. (2005).

One interesting finding of this thesis was that both RA and Hoxa2 have similar effects on OG differentiation and PSA-NCAM expression. As a result, it would be worthwhile to determine if Hoxa2 could act as a downstream effector of RA in oligodendroglial cells. Hence, quantitative RT-PCR analysis should be conducted in order to examine the level of *Hoxa2* mRNA expression in CG4 cells at 8, 16, and 36 hours after RA exposure. In addition, the expression of *Sox9* and *Sox6* should be analyzed as they may be involved in RA's ability to inhibit OG differentiation. As RA has been shown to induce the expression of multiple *Hox* genes in carcinoma cell lines (Simeone et al. 1990; Simeone et al. 1991), the mRNA levels of additional genes, such as *Hoxb1*, *Hoxb2*, and *Hoxa1*, should also be analyzed. This is especially important since there is evidence suggesting that both auto- and cross-regulatory Hox TF loops could affect *Hoxa2* expression (Lampe et al. 2004; Tümpel et al. 2007). Furthermore, as genes located at the 3′ end of the *Hox* clusters are more sensitive to RA than those located at the 5′ end (Simeone et al. 1990), I would suggest treating the CG4 cells after one day with 10 nM RA. This treatment regimen was chosen for two reasons. First, treatment of carcinoma cells with 10 nM RA was found to induce primarily 3′ genes (Simeone et al. 1990). Secondly, treatment of CG4 cells with 10 nM RA after one day was found to not only impair OG differentiation but also reduce PSA-NCAM expression. If RA is found to induce *Hoxa2* expression, further research would then have to be conducted in order to establish a functional relationship between the two.

8.3 Miscellaneous

In addition to RA, several other factors affect *Hoxa2* expression in the hindbrain. For example, FGF8 appears to repress *Hoxa2* expression in r1 (Irving and Mason 2000). Based on this information, as well as our Hoxa2-S CG4 data, it would be expected that treating oligodendroglial cells with FGF8 would promote OG differentiation. However, FGF8 treatment, similar to *Hoxa2* over-expression, inhibits OG differentiation without affecting proliferation (Fortin et al. 2005). In order to resolve this issue, further research is needed to determine if FGF8 treatment actually affects Hoxa2 expression in oligodendroglial cells. In comparison to FGF8, Krox20 induces *Hoxa2* expression in r3

and r5. Although Krox20 is not expressed by oligodendroglial cells (Sock et al. 1997), they do express Krox24 (Sock et al. 1997), which appears to bind to a similar DNA target sequence as Krox20 (Lemaire et al. 1988). Hence, further research, in the form of electrophoretic mobility shift assays and luciferase assays, should be conducted in order to determine if Krox24 could act upstream of Hoxa2 in oligodendroglial cells. Furthermore, as Krox24's role in OG development is currently not known, this information could lead to the identification of potential functions which then could be tested.

9.0 CONCLUSION

The data presented in this thesis is the first to show that Hoxa2 is expressed throughout OG development, diminishing only with the acquisition of a myelinating phenotype. In addition, it is the first to demonstrate a role for Hoxa2 in OG differentiation. Furthermore, it is also the first to identify PSA-NCAM as a downstream target of Hoxa2. As PSA-NCAM affects oligodendroglial migration and myelination, this information suggests that Hoxa2 could also affect these processes. Furthermore, the results suggest that over-expression of *Hoxa2* exhibits similar effects on OG differentiation and PSA-NCAM expression as RA treatment. Based on these findings, further research is needed in order to determine if Hoxa2 could affect migration and/or myelination, as well as whether it could act as a downstream effector of RA in oligodendroglial cells.

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Appendices

Appendix A. Carnegie/Theiler staging [Butler and Juurlink (1987) and Theiler (1972)]

Carnegie Stage	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Theiler Stage	**1st 1/2 12**	**2nd 1/2 12&13**	**14**	**15**	**16**	**17**	**18**
E#	**9**	**9.5**	**10**	**10.5**	**11**	**11.5**	**12**
# Somites (S)	1-3	4-12	13-20	21-29	30-34	35-39; cranial Ss become indistinct	cranial Ss indistinct
Length (mm)	-----	-----	1.2-2.5	1.8-3.3	3.1-3.9	3.5-4.9	5-6
CNS	Open neural plate	Neural folds begin to fuse in cerv.area (7S stage)	Cranial neuropore (NP) closes	Cranial NP closed; posterior NP constricted-still open	Caudal NP closed	Cerebral vesicles distinct	Cerebral Vesicles distinct
Rotation	Lordosis	Early: lordosis; Turning begins (8S stage)	Turning complete; Ventral curvature	-----	-----	-----	-----
Eyes	-----	-----	Lens placode (LP) appears	LP- distinct thickening	Lens pit in older members	Early: lens vesicle- deep pockets Older: pore like	Pore like
Ears	-----	Otic placodes	Otic placode invaginates	Otic vesicle closed in older members	Otic vesicle closed	Otic vesicle closed; develops a short Endolymphatic Duct (ED)	Otic vesicle closed; Elongated ED
Nose	-----	-----	Olfactory placode (OP) appears	OP- distinct thickening	OP indents	Lip around OP	Deepened olfactory pit
Branchial Bars	-----	-----	1 & 2	3 present	1, 2, 3; 3rd & 4th concave	1st bar-mandibular & maxillary	1st bar-mandibular & maxillary
Limb Buds (LB)	-----	-----	cranial LB @ S15 indistinct	Cranial LB distinct @ Ss 8-12	Caudal LB @ Ss 23-28	Elongated LBs curve forward & medially	Elongated LBs curve forward & medially
Tail	-----	-----	-----	-----	Short stump	Tail curves as well	Tail lateral to head
Hair	-----	-----	-----	-----	-----	-----	-----
Other	-----	-----	Spiral form-caudal end lying on R head Narrow long vitelline duct		Cervical sinus appears	-----	Pontine flexure

Carnegie Stage	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**
Theiler Stage	**19**	**20**	**20**	**21**	**21**	**22**	**22**	**23**
E#	**12.5**	**13**	**13.5**	**14**	**14.5**	**15**	**15.5**	**16**
# Somites (S)	Caudal Ss sharply defined	Lumbosacral Ss distinct ; thoracic not	Lumbosacral Ss indistinct	Ss only visible in tail	Ss only Visible in tail	Ss only Visible in tail	Ss only visible in tail	Ss only Visible in tail
Length (mm)	6-7	7-8	8-9	9-10	10-11	10.5-11.5	11-12	12-14
CNS	Cerebral vesicles distinct	Cerebral vesicles Distinct	Cerebral Vesicles distinct	Cerebral Vesicles distinct	Cerebral Vesicles distinct	Cerebral Vesicles distinct	Cerebral Vesicles Distinct	Cerebral Vesicles Distinct
Rotation	-----	-----	-----	------	-----	----	----	----
Eyes	Retinal pigment appears; complete closure of LV	Retinal pigment	Retinal pigment	Eyelids-sm folds-not yet cover eyeball	Lger eyelids Covering most of eyeball	Lger eyelids	Eyelids	Eyelids About to close
Ears	6 auricular hillocks; ED longer	6 auricular hillocks	6 auricular hillocks	Hillocks fuse=pinna	pinna	Pinna covers ~ ½ of external auditory meatus (EAM)	Pinna covers ~ ½ of EAM	Pinna covers >1/2 EAM
Nose	Nostrils narrowed to slits	Nostrils	Nostrils	Nostrils	Nostrils	Nostrils	Nostrils	Nostrils
Branchial Bars	1st bar-mandibular & maxillary	1st bar-mandibular & maxillary	1st bar-mandibular & maxillary	1st bar-mandibular & maxillary	1st bar- mandibular & maxillary	1st bar-mandibular & maxillary	1st bar-mandibular & maxillary	1st bar-mandibular & maxillary
Limb Buds (LB)	Cranial LB-distal rounded footplate & proximal region	Forelimb: footplate has toe rays; hindlimb footplate distinct	fFP:more pronounced toe rays, beginning to crenate; hFP: slight toe rays	fFP-crenated; hFP-toe rays	Fingers separate; hFP-crenation	Fingers distinct; hFP= deep indents but not separate	Distal phalanges separate of toes	Complete Separation of toes; distinct flexures @ elbow &knee
Tail	Longer	Longer	Longer	Longer (curved to left)	Longer	Longer	Longer	Longer
Hair	-----	-----	-----	-----	5 rows of whiskers,primordial hair follicle above ea eye & in front of ea ear	Numerous hair follicles recognized in the skin except head	Numerous hair follicles recognized in the skin except head	Hair Follicles cover entire body
Other	-----	-----	-----	-----	-----	-----	-----	-----

Theiler Stage	**24**	**25**	**26**	**27**
E#	17	18	19	NB
Length	14-18	16.5-20	18-23	23-27
Eyes	Eyelids fused	Eyelids fused & thickened	Eyes barely visible thru lids	Eyes closed
Ears	EAM almost completely covered by pinna			Ears closed
Limbs	Fingers 2-5 parallel; toes still diverge	Fingers & toes parallel		
Hair	Hair follicles over entire body	Short Whiskers	Longer whiskers	
Skin	Becoming wrinkled	Wrinkled & thickened	Markedly wrinkled	
Other	Anterior back almost straight			

Appendix B. Supplemental Figures

Chapter 3

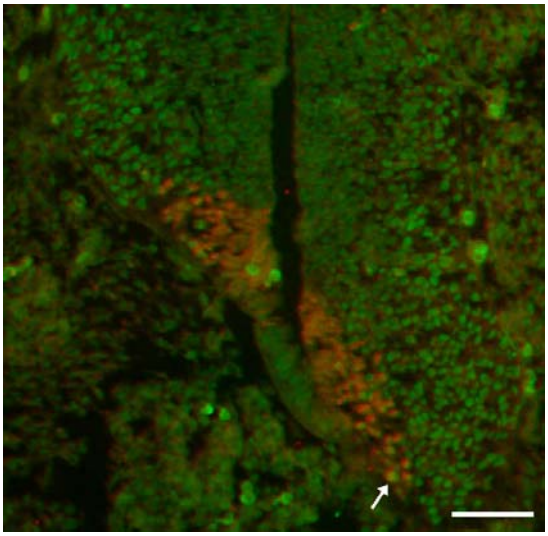

Figure S3.1 Hoxa2 is expressed by some Nkx2.2-immunoreactive cells (arrow) in the mantle layer of the E12.5 spinal cord. [Scale bar = 100 µm]

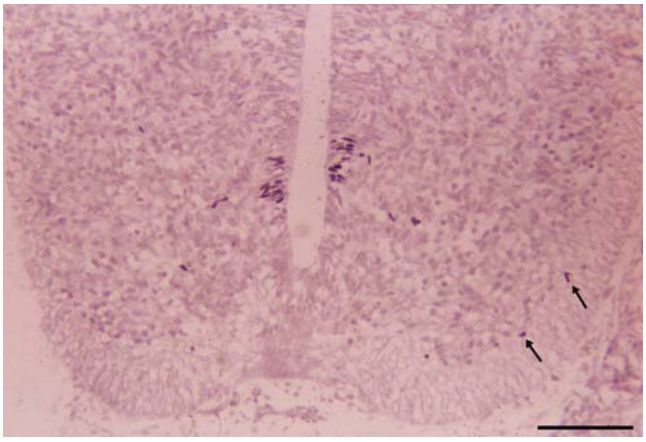

Figure S3.2 At E14.25 Olig2^{+} cells are occasionally seen in the marginal layer (arrows). [Scale bar = 100 µm]

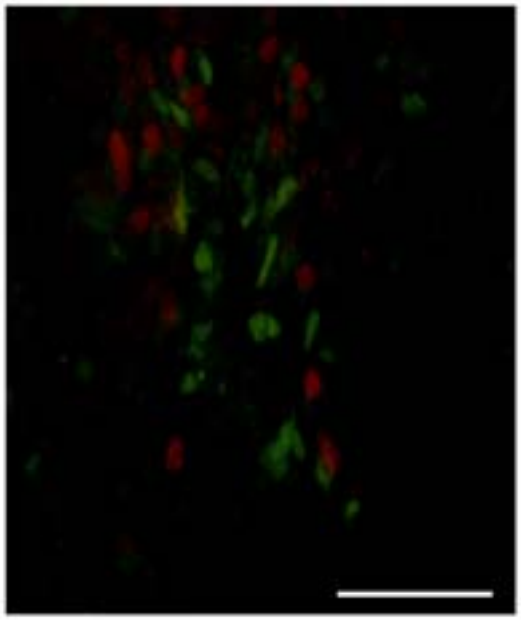

Figure S3.3 There are few if any cells in the E14.25 spinal cord that co-express Nkx2.2 (red) and Olig2 (green). [Scale bar = 100 µm]

Chapter 4

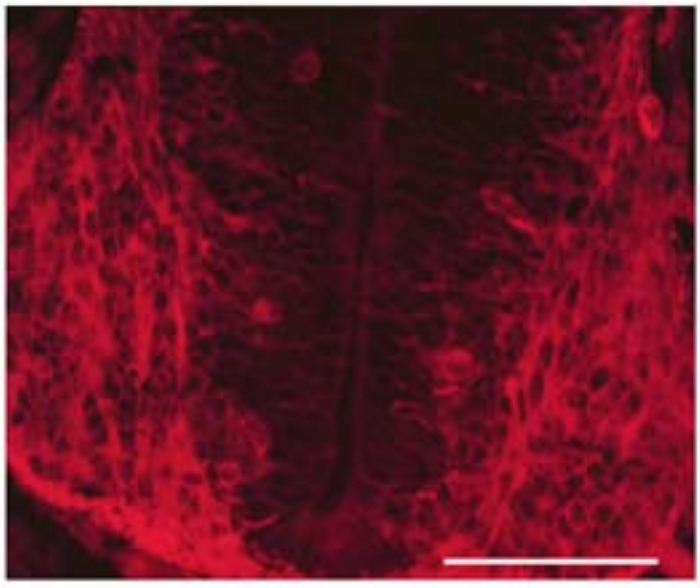

Figure S4.1 β-tubulin expression in the E12.5 spinal cord. [Scale bar = 100 µm]

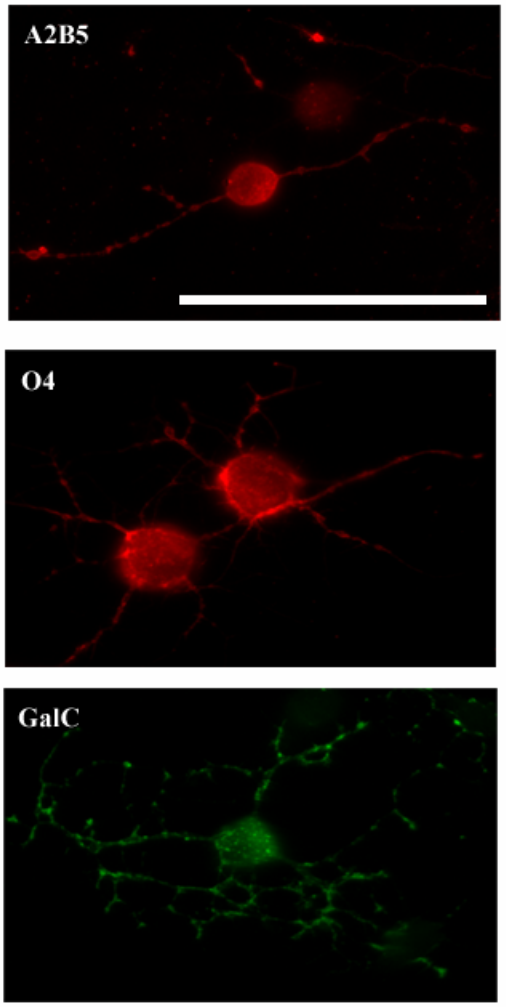

Figure S5.1 CG4 cells exhibit similar morphological and antigenic phenotypes as primary oligodendroglial cells. [Scale bar = 100 µm]

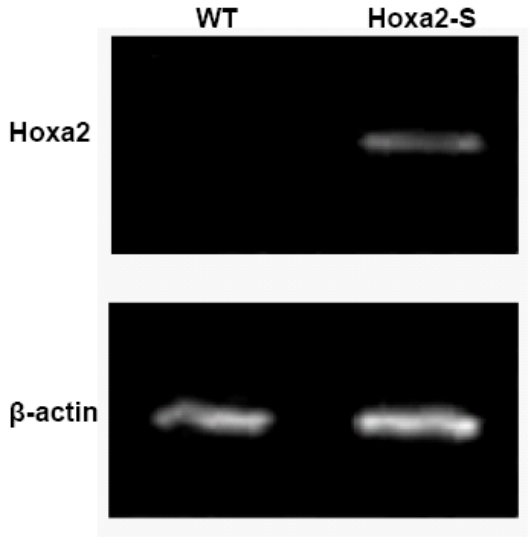

Figure S5.2 Comparison of *Hoxa2* mRNA expression in control and Hoxa2-S CG4 cells via RT-PCR. (*Hoxa2* mRNA expression is evident in control cultures; however, it requires more cDNA to be added to the mixture.)

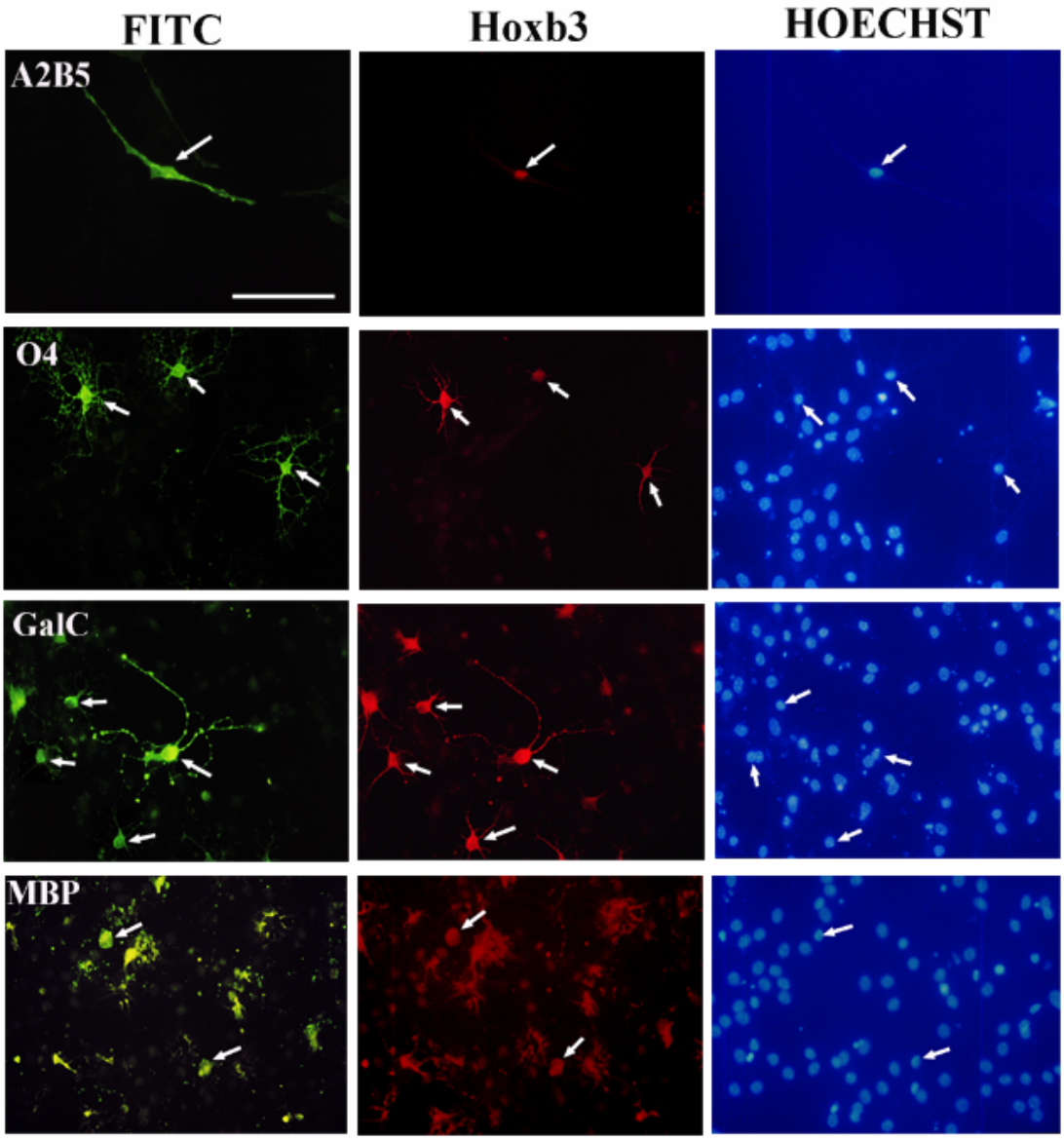

Figure S5.3 Hoxb3 was expressed throughout OG development.

Primary mixed glial cell cultures from neonatal murine cerebral hemispheres were grown in culture for 18 days. Double immunofluorescent staining was conducted with each of the four OG markers [A2B5 (A), O4 (D), GalC (G), and MBP (J)] and Hoxb3 (B, E, H, K). Cells were visualized using nuclear fluorescent Hoechst counterstain (C, F, I, L). Each row illustrates micrographs obtained from individual filters [fluorescein (A, D, G, J), rhodamine (B, E, H, K), and DAPI (C, F, I, L)] of the same field. Arrows demarcate representative Hoxb3 immunoreactive cells. Scale bar = 100μm.

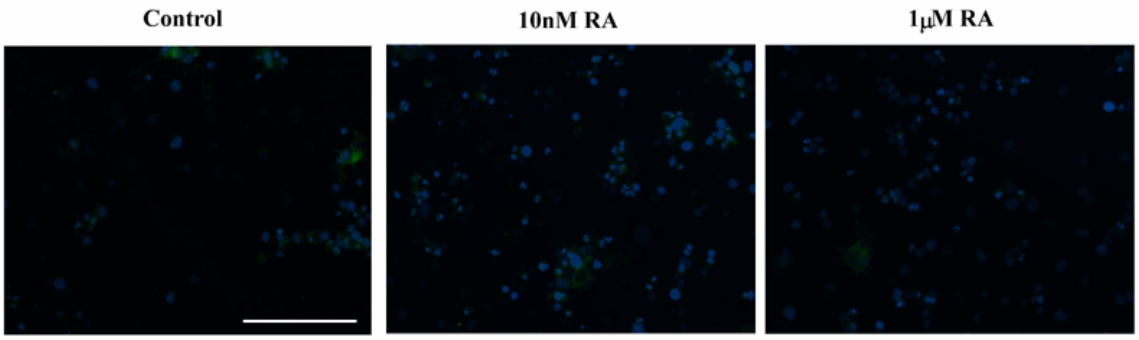

Figure S6.1 Regardless of the experimental condition there are only a few MOG$^+$ cells. [Scale bar = 100 μm]

Appendix C. Published Manuscripts [refer to pg. v for full reference]

Cell Mol Neurobiol
DOI 10.1007/s10571-007-9150-4

ORIGINAL PAPER

Hoxd1 is Expressed by Oligodendroglial Cells and Binds to a Region of the Human Myelin Oligodendrocyte Glycoprotein Promoter in vitro

Jayaum Booth · Danette J. Nicolay · J. Ronald Doucette · Adil J. Nazarali

Received: 21 February 2007 / Accepted: 13 April 2007

Abstract (1) Little information exists on the role of clustered *Hox* genes in oligodendrocyte (OG) development. This study examines the expression profile of Hoxd1 and identifies a potential downstream target in the OG lineage.(2) Immunocytochemical analysis of primary mixed glial cultures demonstrated Hoxd1 was expressed throughout OG development. (3) A human myelin protein gene, myelin oligodendrocyte glycoprotein (MOG), was identified as a putative downstream target of Hoxd1 through Genbank searches utilizing the Hoxd1 homeodomain consensus binding sequence. (4) The dissociation coefficient constant (K_D) and dissociation rate constant (k_d) of the Hoxd1–MOG complex, determined using electrophoretic mobility shift assays (EMSAs), were estimated to be 1.9×10^{-7} M and 1.3×10^{-3} s^{-1}, respectively. The DNA–Hoxd1 homeodomain complex had a half-life ($t_{1/2}$) of 15 min. (5) Mutational analysis of Hoxd1–MOG complexes revealed the binding affinity of M1 (with mutation from $^{-1054}5'$-TAAT-$3'^{-1051}$ to TACT within the consensus binding site) and M2 (with mutation from $^{-1054}5'$-TAATTG-$3'^{-1049}$ to TAATCC within the consensus binding site) probes to the MOG promoter was severely affected. Thus the TAATTG core of the binding sequence appears important for Hoxd1 specificity. (6) Analysis of the involvement of TAAT sites adjacent to the consensus sequence in Hoxd1 binding showed the binding affinity of the M3 probe was affected, but not as severely as the M1 and M2 probes. These in vitro results suggest the TTTAATTGTA sequence is involved in Hoxd1 binding to the MOG promoter but neighboring TAAT sites may also be needed. Thus, MOG may be a target of Hoxd1.

Keywords Oligodendrocytes · Homeodomain · Hoxd1 · DNA-binding · MOG

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Introduction

Oligodendrocytes (OGs) are the cells possessing the ability to assemble a myelin sheath around axons in

the central nervous system (CNS) (Southwood et al. 2004). In culture, OG development has been shown to progress through a number of distinct stages characterized by specific antigenic phenotypes. In particular, during early stages of oligodendrogenesis, $A2B5^+$ oligodendrocyte precursor cells (OPCs) and $O4^+$ pro-OGs actively proliferate (Fok-Seang and Miller 1994) and migrate (Noble et al. 1988). Subsequently, loss of these traits and the emergence of galactocerebroside (GalC) expression (Fok-Seang and Miller 1994; Noble et al. 1988) signal terminal differentiation into premyelinating OGs. Finally, progression to the mature myelinating phenotype occurs when the cells synthesize myelin proteins and elaborate sheet-like membranes (Duchala et al. 1995).

Interestingly, as these cells progress from precursors to mature myelinating OGs, they express a dynamic combination of transcription factors (TFs), which can be divided into specific families. In many cases, multiple members of a particular TF family have been shown to be expressed by oligodendroglial cells. For example, two members of the Hox TF family, Hoxa2, and Hoxb4, have been shown to be expressed throughout oligodendrogenesis in vitro (Nicolay et al. 2004a, b). This family consists of 39 mouse and human *Hox* genes organized into four clusters (Hox A, B, C, D) and located on different chromosomes (Scott 1992; Favier and Dollé 1997; Santagati and Rijli 2003; Akin and Nazarali 2005). These genes, in turn, are characterized by a 180-base pair (bp) homeobox, which encodes a 60-amino acid homeodomain (McGinnis et al. 1984a, b). Through the homeodomain, Hox TFs can regulate the expression of downstream effector genes by binding to specific nucleotide sequences (Hoey and Levine 1988).

In this study, we examined the expression of another Hox TF, Hoxd1, in oligodendroglial cells. As with Hoxa2 and Hoxb4, Hoxd1 expression was found throughout OG development in vitro. Interestingly, a Hoxd1 consensus binding sequence was subsequently identified in the human myelin oligodendrocyte glycoprotein (MOG) promoter. Equilibrium and kinetics studies carried out with electrophoretic mobility shift assays (EMSAs) showed specific affinity (K_D, 1.9×10^{-7} M; K_d, 1.3×10^{-3} s^{-1}; $t_{1/2}$, 15 min) of the Hoxd1 homeodomain for the MOG promoter region. Mutational analysis with EMSA showed the proposed Hoxd1 binding site is important for the binding affinity of the protein to the MOG promoter region, but adjacent TAAT sites may also be involved. Hence, these in vitro results suggest Hoxd1 could play a role in OG differentiation and/or maturation via affecting the expression of MOG.

Materials and Methods

Primary Glial Cultures

Cerebral hemispheres from newborn mice were dissected in Dulbecco's modified Eagle's medium/F12/10% fetal bovine serum (DMEM/F12/10% FBS). Subsequently, single cell suspensions, obtained by forcing diced tissue through a 75-μm Nitex mesh, were plated in DMEM/F12/10% FBS for 4 days to allow adherence of the cells. They were then cultured for two sequential 1-week periods, first in a B104 conditioned medium, then in a low serum-containing (0.3%) growth medium (GP medium) [described previously by Gard and Pfeiffer (1990) and Doucette and Devon (1994)] supplemented with 10 ng/ml basic fibroblast growth factor (bFGF). Culture medium was changed every 4 days.

Immunocytochemistry

Immunocytochemistry utilized the following primary antibodies: anti-Hoxd1 (1:200), A2B5 (1:10, hybridoma; American Type Cell Culture [ATCC]), O4 (1:10, hybridoma; Sommer and Schachner 1981), anti-galactocerebroside (GalC) (1:20, hybridoma; Ranscht et al. 1982), and anti-myelin basic protein (MBP) (1:800; Sternberger Monoclonals, Lutherville, MD). The secondary antibodies used included goat anti-mouse IgM fluorescein isothiocyanate (FITC) (1:100, A2B5/ O4; Sigma, Oakville, ON), donkey anti-mouse IgG FITC (1:50, GalC; BIO/CAN Scientific, Mississauga, ON), and goat anti-rabbit IgG CY3 (1:200, Hoxd1; BIO/CAN Scientific). Double labeling Hoxd1 with A2B5, O4, GalC, or MBP was conducted using a procedure previously described in Nicolay et al. (2004a, b) with the following modifications: cells were incubated for 4 h at room temperature (RT) in anti-Hoxd1 diluted in 1% SM/0.03% TX. The cells' nuclei were stained using Hoechst dye (Sigma, ON).

Polymerase Chain Reaction (PCR)

All PCR amplifications, which utilized the Perkin Elmer GeneAmp^{R} PCR kit with AmpliTaq^{R} polymerase, were conducted with a DNA thermal Cycler (Perkin Elmer, Applied Biosystems, Mississauga, ON).

Amplification of the Hoxd1 Gene Fragment

PCR was employed to clone a 327 bp sequence (696–1023 bp) using the Hoxd1 gene as the DNA template (Nazarali et al. 1992). The amplified gene fragment consists of the N-terminal region just after the hexapeptide domain, the homeodomain, and the entire C-terminal region of the *Hoxd1* gene (Nazarali et al. 1992). Amplifications, performed using primer A and primer B (listed in the *Plasmid and oligonucleotides* section below), consisted of an initial denaturation at 94°C for 3 min followed by 30 cycles of 1 min at 94°C, 2 min at 58°C, 1 min at 72°C, and a final extension of 10 min at 72°C. The PCR-amplified *Hoxd1* DNA fragment was digested with *Eco*RI and *Xho*I and subcloned into the pFLAG-2 expression vector (Sigma, St. Louis, MO) inframe with the methionine codon. The *Hoxd1* clone was sequenced using a T7 Sequenase $_{v2.0}$ kit (Amersham Pharmacia Biotech, Piscataway, NJ) to confirm the sequence of PCR-amplified DNA.

Expression and Purification of the Hoxd1 Homeodomain Protein

Transformed *E. coli* XL1-Blue Supercompetent cells (Stratagene, La Jolla, CA) containing the pFLAG-*Hoxd1* plasmid were grown in LB medium containing ampicillin (5 μg/ml) at 37°C. The Hoxd1 C-term protein was then isolated using an established protocol (Hoey 1990) and purified as described by Kumar and Nazarali (2001). The protein was quantified using the Bio-Rad DC protein assay (Bio-Rad laboratories, Hercules, CA), and the yield of purified Hoxd1 homeodomain protein was found to be approximately 100 μg/l of culture.

Electrophoretic Mobility Shift Assays (EMSA)

Protein–DNA complexes were resolved by EMSA as described elsewhere (Ekker et al. 1991; Phelan et al. 1995; Kumar and Nazarali 2001) with the following modifications: (a) Binding reactions were performed in a total volume containing: Labeled probe (50,000 cpm), purified Hoxd1 protein, 1× binding buffer (10 mM HEPES, pH 7.9; 1 mM $MgCl_2$; 60 mM KCl; 0.5 mM EDTA; 1 mM DTT; 10% glycerol), and 1.0 μg poly dI-dC. (b) Samples were incubated for 30 min at room temperature and then separated on 8% non-denaturing polyacrylamide gel (1.5 mm thickness) at 4°C in 0.25× Tris–borate–EDTA (TBE) buffer for 1.5 h at 140 V. (c) Gels were fixed, dried, and visualized by autoradiography. The complex protein–DNA was sliced out of the dry gel and counts taken from the scintillation counter. In experiments that contained competitor DNA, excess unlabeled oligonucleotide over the labeled probe was included. Nuclear extracts from embryonic mice at 12 days (E12) post coitum were prepared as described by Lahiri and Ge (2000). Five μg of nuclear extracts was used for each EMSA reaction. The anti-Hoxd1 antibody was added to specific reactions at a concentration of 1:500 to the mixture of nuclear extracts and DNA probe.

Competition Assay

Competition assays involved the use of cold probes including the wild type probe (Normal), and M1 to M3 mutant probes containing a specific change in the 41 bp region of the MOG promoter (see below). Concentrations of 0, 5×, 50×, and 150× of cold probe to hot normal probe were used individually and EMSAs were performed.

Plasmid and Oligonucleotides

All oligonucleotides used in this study were purchased from Invitrogen-GIBCO-BRL (Burlington, Ontario, Canada). The target MOG oligonucleotide encompassing the −1072 to −1032 region of the promoter (N) and the mutated MOG probes (M1 to M3) were as follows:

MOG (N) 5′-CAAATTTTTAATTTTA**TTTAATTGTA**ATTAATTTTAAGTGG-3′

MOG (M1) 5′-CAAATTTTTAATTTTA**TTTA*C*TTGTAATTAATTTTAAGTGG-3′**

MOG (M2) 5′-CAAATTTTTAATTTTA**TTTAAT*CC*TA**ATTAATTTTAAGTGG-3′

MOG (M3) 5′-CAAATTTTTA***C***TTTTA**TTTAATTGTA*C***TTA***C***TTTTAAGTGG-3′

The primers used to amplify Hoxd1 were:

Primer A 5′-TCGAATTCCTGTCCGAATATGG-AGCC-3′

Primer B 5′-TGCTCGAGGGAAGGCTCTTGAG-CCTG-3′

Single-stranded oligonucleotides used in EMSA were annealed by heating equal molar amounts of oligonucleotides in STE buffer (10 mM Tris pH 8.0, 50 mM NaCl, 1 mM EDTA) above their denaturing temperature (94°C), followed by slow cooling to an ambient temperature. The oligonucleotides were then stored at 4°C overnight before use in EMSA.

Results

Expression by Oligodendroglial Cells

Immunocytochemical analysis of primary mixed glial cell cultures demonstrated Hoxd1 was expressed throughout OG development (Fig. 1). In particular, Hoxd1 expression was evident in A2B5, O4, GalC, and MBP-immunoreactive oligodendroglial cells. Although the location of immunoreactivity was primarily nuclear, cytoplasmic staining was also observed at later stages of oligodendrogenesis. In addition, Hoxd1 expression did not appear to be limited to OGs as demonstrated by the expression of Hoxd1 in cells that were negative for the aforementioned oligodendroglial markers.

Hoxd1 Protein Interacts with the Human MOG Promoter

Utilizing the recently characterized optimal DNA-binding site sequence of the Hoxd1 homeodomain, potential downstream targets were searched through GenBank. One putative downstream target identified by this search was the myelin protein, MOG. In particular, the Hoxd1 consensus-binding site for *MOG* was located in the promoter region of the gene from 1047 bp to 1057 bp upstream of the start codon. Subsequently, a double stranded oligonucleotide, which represented the sequence from nucleotide position −1072 to −1032 within the MOG promoter and contained the putative Hoxd1 binding sequence, ATTTAATTGTA (Kumar and Nazarali 2001), was synthesized and used as a probe in EMSA to determine its binding characteristics with purified recombinant Hoxd1. As shown in Fig. 2, the presence of a single DNA–protein complex was observed when the probe was incubated with Hoxd1 (lane H), but not with the control bovine serum albumin (lane C). The presence of 10, 20, and 50-fold excess of unlabeled wild type MOG promoter oligonucleotide in the reaction competed efficiently for the formation of the complex, as the intensity of the band decreased with increasing fold of the unlabeled competitor and was completely blocked after addition of 50-fold excess cold probe (Fig. 2, lane 3, 4, 5).

Hoxd1 Protein Specificity for MOG Promoter

We conducted gel retardation assays (Ekker et al. 1991; Florence et al. 1991; Iler and Abate-Shen 1996) to quantify the binding affinities of Hoxd1–MOG complexes. The dissociation coefficient constant (K_D) of Hoxd1 was determined by using a fixed amount of probe with increasing concentration of the protein. We determined a K_D in the order of 1.9×10^{-7} M for the Hoxd1–MOG complexes from a plot of fraction bound vs. Hoxd1 homeodomain concentration (nM) (Fig. 3A, B); K_D was estimated to be the protein concentration that produced half maximal protection (Ekker et al. 1991; Kumar and Nazarali 2001).

The dissociation rate constant (k_d), a measure of the stability of the complex between Hoxd1 and the MOG promoter sequence, was calculated from kinetic studies (Fig. 4A, B). Using the formula ln (fraction DNA bound) = $-k_d t$ (Ekker et al. 1991; Kumar and Nazarali 2001), we determined a k_d of 1.3×10^{-3} s^{-1}. The time required for half of the DNA–protein complex to dissociate, defined as the half-life ($t_{1/2}$), was calculated using the formula $t_{1/2} = -\ln(0.5)/k_d$ (Ekker et al. 1991; Kumar and Nazarali 2001) and found to be 15 min.

Effect of MOG Promoter Mutation on Binding Specificity of Hoxd1 Protein

To determine whether the core $^{-1054}$5′-TAATTG-3′$^{-1049}$ site within the Hoxd1 binding site on the MOG promoter, as well as three other TAAT sites flanking the putative binding site, were required for binding Hoxd1, we synthesized probes M1 to M3 and performed EMSAs with each probe. The M1 probe contained a one-nucleotide change from normal probe ($^{-1054}$5′-TAAT-3′$^{-1051}$ to TACT) within the

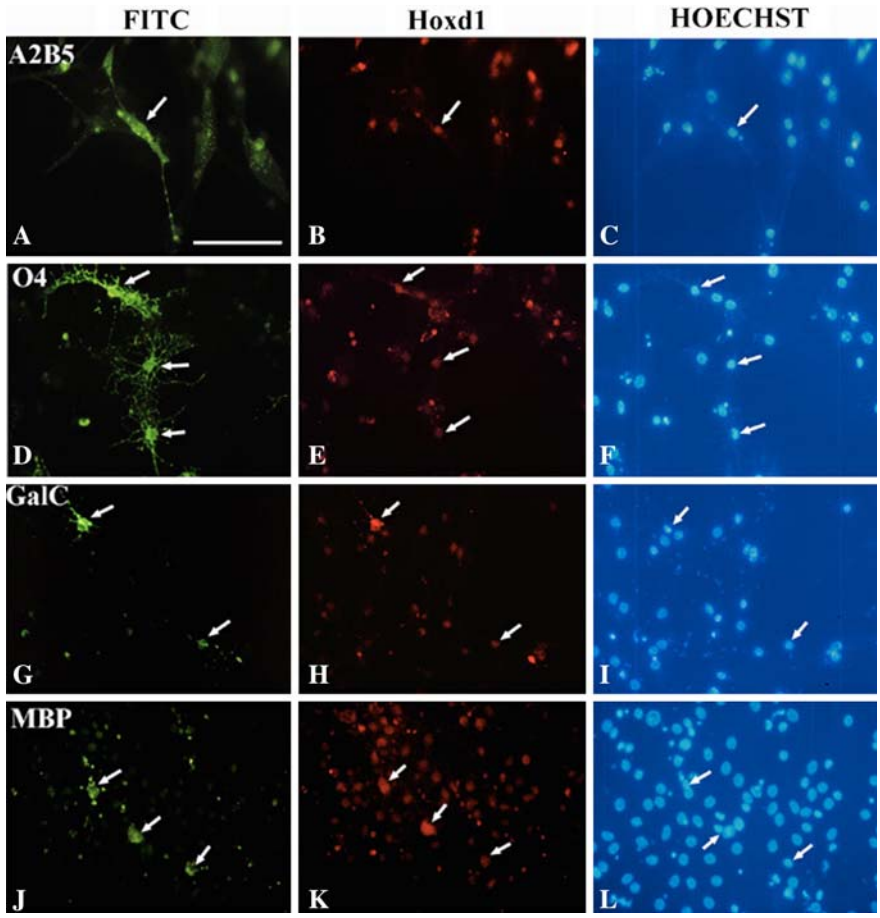

Fig. 1 Hoxd1 is expressed throughout oligodendrocyte development. Primary mixed glial cells were grown in culture for a total of 18 days. Double immunofluorescent staining was performed with Hoxd1 (**B**, **E**, **H**, **K**) and one of the four OG markers [A2B5 (**A**), O4 (**D**), GalC (**G**), MBP (**J**)]. The nuclei were visualized utilizing HOECHST fluorescent counterstain. Each row illustrates micrographs obtained from individual filters [fluorescein (**A**, **D**, **G**, **J**), rhodamine (**B**, **E**, **H**, **K**), and DAPI (**C**, **F**, **I**, **L**)] of the same field. Arrows identify representative Hoxd1 immunoreactive oligodendroglial cells. Bar = 100 μm for Fig. (**A**–**L**)

putative Hoxd1 binding site. The EMSA results (Fig. 5A, B) establish that Hoxd1 binds to the MOG promoter with the intact TAAT site, but poorly with the M1 probe (TACT). Thus, Hoxd1 protein binding to the MOG promoter requires this TAAT site. Similarly, the M2 probe, which contained a two-nucleotide change from normal probe ($^{-1054}$5′-TAATTG-3′$^{-1049}$ to TAATCC) within the consensus-binding site, also bound poorly to Hoxd1 protein (Fig. 5A, B). Therefore, the Hoxd1 protein appears to require the TG nucleotide just after the TAAT site for efficient binding to the MOG promoter sequence. The M3 probe had a one-nucleotide change on three TAAT sites (TAAT to TACT at positions −1041 to −1044, −1045 to −1048, and −1061 to −1064) flanking the consensus-binding site. Results show the binding of M3 to the Hoxd1 protein decreases, but not as significantly as noted for M1 and M2. Thus, these results suggest the ATTTAATTGTA binding site is involved in the in vitro binding specificity of Hoxd1 to MOG. However, one or more of the flanking TAAT sites may also play a role in the binding of Hoxd1.

EMSA was performed with different ratios of cold to hot normal probe (0, 5×, 50×, 150×). Similarly, each oligonucleotide (M1 to M3) was used as a cold probe in EMSA. As shown in Fig. 6(A), the presence of a single DNA–protein complex is observed when the probe is incubated with the Hoxd1 protein (lane 2), but not with control bovine serum albumin (lane 1). The presence of 50-fold and 150-fold excess of the unlabeled wild type (normal) MOG promoter oligonucleotide (lane 8 and 9, respectively) in the reaction competed efficiently for the formation of the complex. Similarly, 50× or 150× excess cold probe (M1, M2, M3) is sufficient to out-compete hot normal probe in each assay. At 5× excess cold probe, N cold competitor (Fig. 6A, lane 7), and M3 (Fig. 6B, lane 7) have almost the same intensity band, although M3 appears slightly more intense. However, M1 (Fig. 6A,

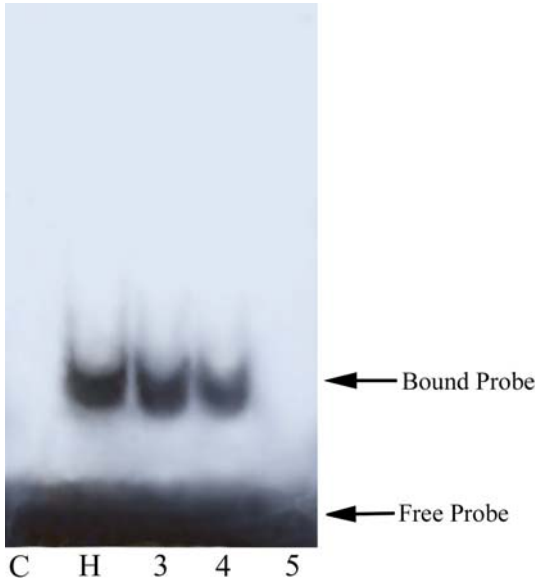

Fig. 2 EMSA shows the Hoxd1 homeodomain can bind to the MOG promoter region causing a shift. C = Control (BSA protein in reaction mixture); H = Hoxd1 protein in reaction mixture. The competition assay shows the probe is binding to the protein. As the concentration of cold probe increases, the shift band intensity decreases until it disappears. Lane 3 is 10× the amount of cold probe to hot; Lane 4 is 20× the amount of cold probe to hot; Lane 5 is 50× the amount of cold probe to hot

lane 3) and M2 (Fig. 6B, lane 3) have more intense bands than both M3 and N probes. This suggests M1 and M2 are unable to compete efficiently at this ratio of cold probe to hot probe, and the core binding sequence involved in Hoxd1 binding to the MOG promoter is TAATTG.

Hoxd1 Protein Binds to MOG in Embryonic Mice Cells

In addition to establishing that Hoxd1 recombinant protein binds to MOG, we determined Hoxd1 binding activity to MOG promoter in nuclear extract to confirm our results. Nuclear extract from embryonic mice at 12 days (E12) post coitum was used in EMSA experiments. As shown in Fig. 7, a single large band representing the MOG–protein complexes is formed (lanes 3–6). Furthermore, increasing the protein concentration in the nuclear extract results in an increased intensity of the bands (lanes 3–6). These results, therefore, suggest homeodomain proteins/cofactors in nuclear extract bind to the MOG promoter sequence.

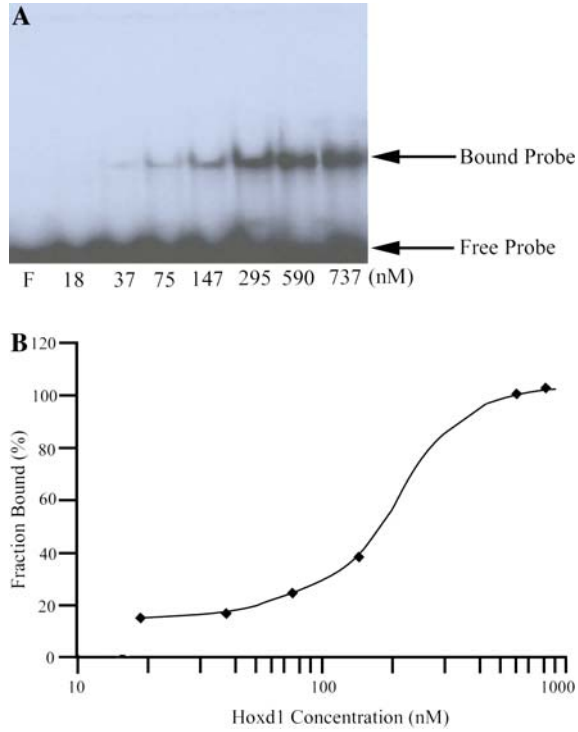

Fig. 3 (**A**) The affinity of the MOG promoter to the homeodomain protein was measured by EMSA. Reactions contained a fixed amount of labeled oligodeoxynucleotide and increasing concentrations of Hoxd1 homeodomain. Bound and free DNA were visualized by autoradiography. F, free probe. (**B**) % fraction of bound DNA as a function of the log of the Hoxd1 homeodomain protein concentration. The dissociation coefficient constant (K_D;1.9×10^{-7} M) was estimated as the concentration of half-maximal binding under conditions of excess protein when a plateau was reached and saturation of DNA has occurred (Ekker et al. 1991)

In order to establish if Hoxd1 may be one of the binding proteins in the nuclear extract, we performed EMSA in the presence of antibodies directed against Hoxd1 protein. The use of pre-immune serum and labeled N probe did not result in a band shift or formation of any specific complexes (Fig. 8, lane 1), although the addition of the anti-Hoxd1 antibody stopped the formation of specific complexes (Fig. 8; lane 2 compared with lane 4).

Discussion

Hoxd1, a member of the labial subfamily of Hox TFs, was recently shown to be expressed in the embryonic murine telencephalon (Wolf et al. 2001), which is

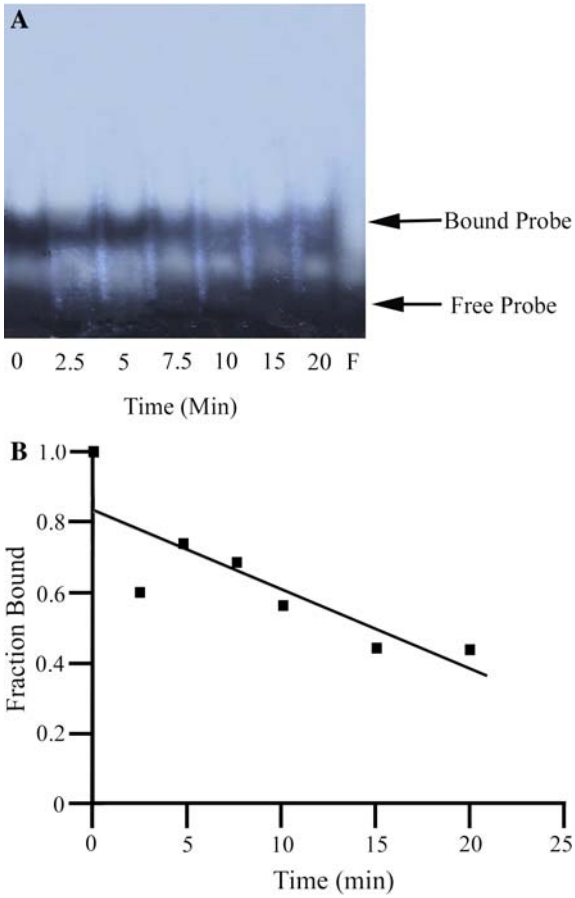

Fig. 4 (**A**) EMSA showing the decay of the homeodomain Hoxd1–DNA complexes as a function of time. DNA–Hoxd1 complexes were incubated with cold probe in competition reactions at time indicated. Lane 1–7= 0, 2.5, 5, 7.5, 10, 15 and 20 min. 0 min indicates binding reactions were loaded immediately on gel after addition of cold competitor. F, free probe with no protein. (**B**) Data were plotted as a function of ln (fraction DNA bound) against various time (min) intervals. The dissociation rate constant, k_d, was calculated using the equation: ln (fraction DNA bound) = $-k_d t$ (Ekker et al. 1991)

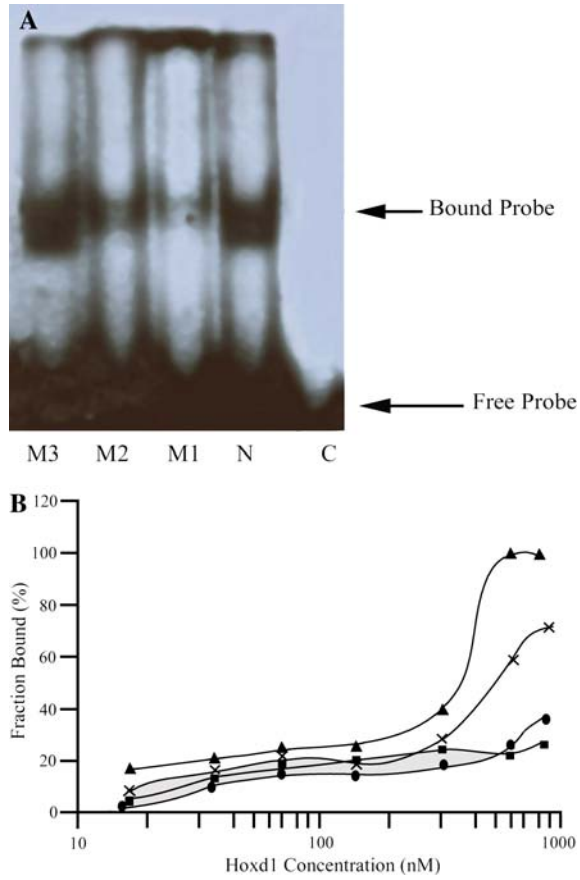

Fig. 5 (**A**) EMSA was performed using normal (N) and mutated (M1, M2, and M3) probes with the same molar concentration of probe (50,000 cpm) and Hoxd1 protein (400 nM). The N probe binds strongly to Hoxd1, whereas M1 and M2 bind very weakly to the probe. M3 binds stronger than M1 or M2. C = Bovine serum albumin protein + labeled normal (N) MOG probe. (**B**) Comparison between normal and three mutated MOG promoter elements for affinity of Hoxd1 binding. These results suggest TAAT and TG sites within the consensus Hoxd1 binding site are involved significantly in the specific binding of Hoxd1. Line connected by triangles (▲) represents the fraction of normal (N) probe bound to the Hoxd1 protein. Line connected by crosses (×) represents the fraction of mutated (M3) probe bound to the Hoxd1 protein. Line connected by circles (●) represents the fraction of mutated (M1) probe bound to the Hoxd1 protein and line connected by squares (■) represents the fraction of mutated (M2) probe bound to the Hoxd1 protein. (See methods and materials for details of probes used; N, M1 to M3)

known to be populated by OPCs (Kessaris et al. 2006). Therefore, in the present work, Hoxd1 expression was analyzed in primary mixed glial cultures obtained from the cerebral hemispheres of newborn mice. These cultures consisted of oligodendroglial cells grown on top of an astrocytic monolayer. The results from this experiment show that although Hoxd1 is expressed throughout OG development, the location of immunoreactivity changed with differentiation. In particular, cytoplasmic Hoxd1 expression was seen in premyelinating and myelinating OGs. This cytoplasmic staining was not expected although it has also been reported with other TFs (Armstrong et al. 1995; Nicolay et al. 2004a, b; Wang et al. 2001).

Subsequently, an extensive search of Genbank utilizing the Hoxd1 homeodomain consensus binding site (Kumar and Nazarali 2001) identified the human

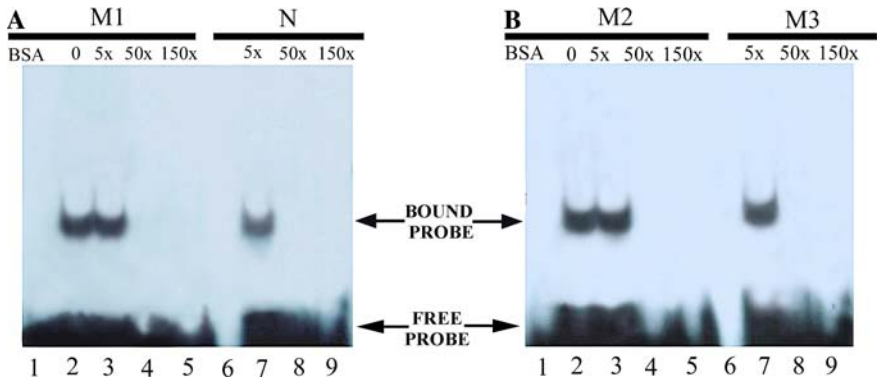

Fig. 6 (**A**, **B**) Competition assays were performed using 50,000 cpm of labeled MOG (N) promoter oligodeoxynucleotide and 400 nM of purified Hoxd1 homeodomain protein in each reaction except for lanes containing 100 ng of BSA as control (**A**: Lanes 1; **B**: Lane 1). Unlabelled competitor oligodeoxynucleotides (N, M1, M2 or M3) were added in 5×, 50×, 150× excess to the hot probe (see text for details)

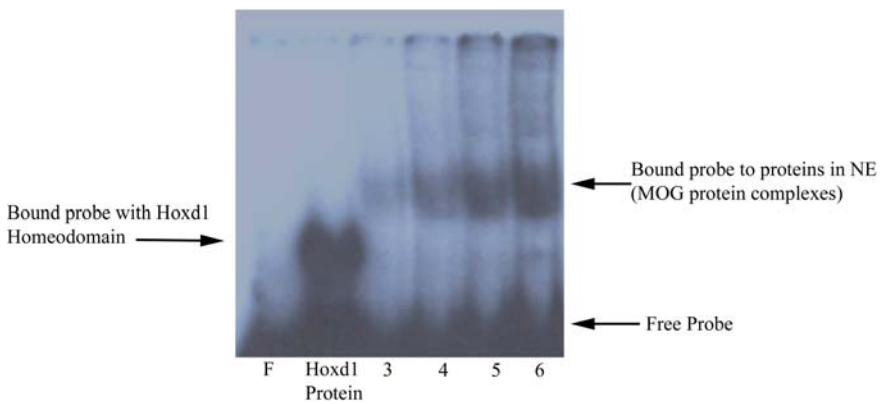

Fig. 7 EMSA was performed using E12 nuclear extract. A single band was formed, representing the MOG–protein complexes. With increasing concentration of E12 nuclear extract (1×, 2×, 3×, 4×), the MOG-protein band increases in intensity. Lane 1, F is free probe; Lane 2, Hoxd1 homeodomain protein bound to N probe; Lane 3 is E12 nuclear extract (3.5μg); Lane 4 is E12 nuclear extract (7 μg); Lane 5 is E12 nuclear extract (10.5 μg); Lane 6 is E12 nuclear extract (14 μg). NE, nuclear extract. MOG–protein complexes represent proteins in NE that bind to the MOG promoter sequence (N)

myelin protein gene, MOG, as a potential downstream target of Hoxd1. MOG, which is a component of myelin protein in the CNS, is expressed on the surface of oligodendroglial processes and myelin sheaths (Brunner et al. 1989; reviewed in Johns and Bernard 1999). Interestingly, reports indicate MOG is alternatively spliced in humans (Ballenthin and Gardinier 1996; Pham-Dinh et al. 1995) but not mice (Ballenthin and Gardinier 1996).

The binding kinetics of the Hoxd1 protein to a 41 base pair region of the human MOG promoter containing the Hoxd1 consensus sequence were examined using EMSAs. From these mobility shift experiments, the equilibrium dissociation coefficient (K_D) for Hoxd1 homeodomain at physiological ionic strength was estimated to be 1.9×10^{-7} M. This K_D value for the recognition of the MOG promoter containing the core TTTAATTGTA binding sequence indicates a relatively weak affinity as compared to the K_D reported for other homeodomains. The absence of the N-terminal in our Hoxd1 protein may have contributed to the low-binding affinity to MOG. In particular, Gehring et al. (1994) suggest the DNA binding constant of a truncated homeodomain may be reduced about 10-fold relative to the intact homeodomain. This reduced binding affinity can be

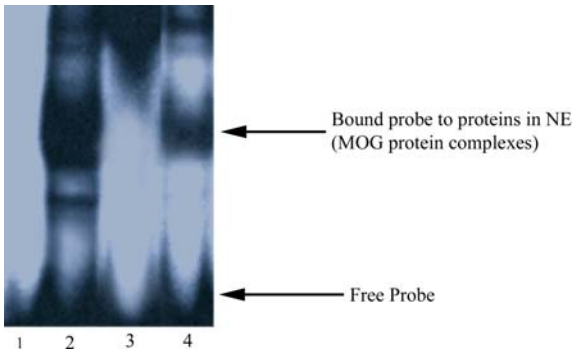

Fig. 8 This supershift experiment determined that proteins other than Hoxd1 bind to the normal (N) MOG promoter sequence in E12 nuclear extract (NE). Lane 1, pre-immune serum + labeled N probe. Lane 2, nuclear extract of E12 mice with labeled probe; Lane 3, Anti-Hoxd1 + labeled probe; Lane 4, nuclear extract of E12 mice with labeled N probe and anti-Hoxd1 antibody. NE, nuclear extract. MOG–protein complexes represent proteins in NE that bind to the MOG promoter sequence (N)

attributed to the absence of the DNA contacts formed by the N-terminal arm of the intact homeodomain in the minor groove.

The low affinity of the recombinant Hoxd1 for the MOG promoter may also be due to its dependence on cofactors not present in the EMSA reaction. Hoxd1 sequence contains an interaction motif (FEWMK) that may allow it to interact with cofactors such as Pbx in the same fashion as other members of the family. In EMSA experiments where E12 nuclear extract is used, the presence of cofactors and/or other homeobox proteins may contribute to the affinity of Hoxd1 to the MOG promoter. Interestingly, the K_D for Hoxd1 as determined by Kumar and Nazarali (2001) was 8.6×10^{-9} M, which is higher than the K_D reported here. The use of a single Hoxd1 binding site core as compared to multiple concatamer binding sites in Kumar and Nazarali (2001) experiments may have contributed to at least a 10-fold decrease in the binding affinity of Hoxd1.

The MOG promoter sequence, which was used for kinetic and equilibrium studies encompassing the Hoxd1 consensus binding sequence TTTAATTGTA, was subjected to one or two base alterations. The results of the dissociation coefficient equilibrium studies from mutant probes indicate oligonucleotides containing the ATTTA***C***TTGTA and ATTTAAT***CC***TA core sequences exhibit very weak affinities for Hoxd1 protein. A change in the binding site by one base, from A to C in M1, and two bases, from TG to CC in M2, significantly decreased the affinity of Hoxd1 for the target sequence. Although the change in the three other TAAT sites (TAAT to TACT; M3 probe) flanking the core-binding site resulted in a several fold decrease in binding affinity compared to the normal probe, the M3 probe had a higher affinity when compared to the mutational change within the core binding site. Thus, the TAATTG sequence seems to be important for Hoxd1 binding, but neighboring TAAT sites may also play a role. Results of the competition assay suggest M1 and M2 mutants are involved in Hoxd1 binding to the MOG promoter region and, therefore, confirm the equilibrium results. In contrast, the M3 probe competed similarly to the normal probe, demonstrating the TAAT sites adjacent to the core sequence in the MOG promoter play a minimal role in Hoxd1 binding in vitro.

In conclusion, Hoxd1 has been shown to be expressed by oligodendroglial cells and binds to a specific nucleotide sequence in the human MOG promoter. These results suggest Hoxd1 may play a role in OG differentiation and/or maturation. However, further research will be needed to determine whether Hoxd1 inhibits or promotes this process in oligodendroglial cells.

Acknowledgement Supported by research grants from the Canadian Institutes of Health Research (CIHR) and the Multiple Sclerosis Society of Canada to AJN and JRD.

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GLIA 48:14–26 (2004)

Early Stages of Oligodendrocyte Development in the Embryonic Murine Spinal Cord Proceed Normally in the Absence of *Hoxa2*

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KEY WORDS Olig2; Nkx2.2; Pax6; Hoxa2; oligodendrocyte specification; primary glial cultures

ABSTRACT Recent discoveries have enhanced our knowledge of the transcriptional control of oligodendrocyte (OG) development. In particular, the transcription factors (TFs) Olig2, Pax6, and Nkx2.2 have been shown to be important in the specification and/or maturation of the OG lineage. Although numerous other TFs are expressed by OGs, little is known regarding their role(s) in oligodendrogenesis. One such TF is the homeobox gene *Hoxa2*, which was recently shown to be expressed by $O4^+$ pro-oligodendrocytes. The objectives of this study were to examine the expression of Hoxa2 during the early stages of OG development, as well as to determine whether *Hoxa2* is required for specification and/or early maturation of OGs. Immunocytochemical analysis of primary mixed glial cultures demonstrated that Hoxa2 was expressed throughout oligodendrogenesis, diminishing only with the acquisition of a myelinating phenotype. Serial transverse spinal cord sections from embryonic days 12.5, 14.25, 16, and 18 $Hoxa2^{+/+}$, $Hoxa2^{+/-}$, and $Hoxa2^{-/-}$ mice were subjected to single and double immunohistochemical analysis in order to examine Hoxa2, Olig2, Nkx2.2, and Pax6 expression profiles. Results obtained from $Hoxa2^{+/+}$ and $Hoxa2^{+/-}$ mice suggested that Hoxa2 was expressed by migratory oligodendroglial cells. In addition, comparison of spinal cord sections obtained from $Hoxa2^{+/+}$, $Hoxa2^{+/-}$, and $Hoxa2^{-/-}$ mice suggested that specification and early maturation of OGs proceeded normally in the absence of *Hoxa2*, since there were no obvious alterations in the expression patterns of Olig2, Nkx2.2, and/or Pax6. Hence, although Hoxa2 is expressed throughout OG development, it does not appear to be critical for early stages of oligodendrogenesis in the murine spinal cord.

INTRODUCTION

Oligodendrocyte precursor cells (OPCs), which are characterized by platelet-derived growth factor-α-receptor (PDGFαR) expression, first appear in the murine spinal cord at embryonic day 12.5 in a distinct region of the ventral ventricular zone (vVZ) (Sun et al., 1998). This region has been shown to correspond to the motor neuron progenitor (pMN) domain, which is one of five ventral progenitor domains formed in response to graded Sonic hedgehog (Shh) concentrations (Ericson et al., 1997; Briscoe et al., 2000; Novitch et al., 2001; Fu et al., 2002). In particular, Shh, which is expressed and secreted by cells of the notochord and floorplate, initially regulates the expression of the transcription factors (TFs) that define the ventral (Nkx2.2/

Grant sponsor: Canadian Institutes of Health Research; Grant sponsor: Multiple Sclerosis Society of Canada; Grant sponsor: Rx&D/CIHR.

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Received 23 August 2003; Accepted 18 December 2003

DOI 10.1002/glia.20028

Published online 30 April 2004 in Wiley InterScience (www.interscience.wiley.com).

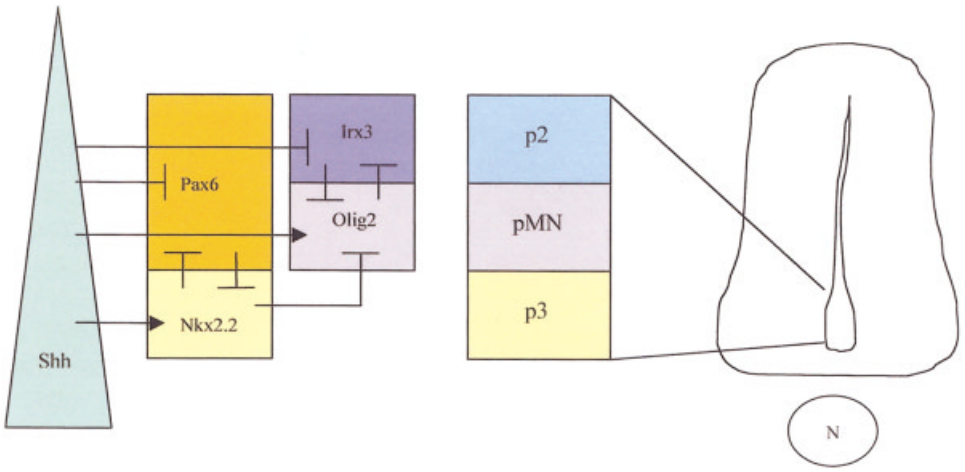

Fig. 1. Platelet-derived growth factor-α-receptor (PDGFαR)$^+$ oligodendrocyte precursor cells (OPCs) originate from the pMN domain. Sonic hedgehog (Shh), which appears to exhibit a ventral to dorsal decreasing concentration gradient, represses (⊥) and enhances (→) the expression of class I (Pax6 and Irx3) and II (Nkx2.2 and Olig2) transcription factors (TFs), respectively, that define the boundaries of the pMN domain. These boundaries are further maintained and refined by repressive interactions between these TFs (Echelard et al., 1993; Marti et al., 1995; Ericson et al., 1997; Briscoe et al., 2000; Novitch et al., 2001; Fu et al., 2002). N, notochord.

Pax6) and dorsal (Olig2/Irx3) boundaries of the pMN domain (Echelard et al., 1993; Bumcrot et al., 1995; Marti et al., 1995; Ericson et al., 1997; Briscoe et al., 2000; Novitch et al., 2001) (Fig. 1). Subsequently, repressive interactions between these TFs maintain and refine these boundaries (Briscoe et al., 2000; Novitch et al., 2001).

Olig2, Pax6, and Nkx2.2 have been shown to be important in OPC specification and/or maturation, since mutations of these genes lead to anomalies in these processes (Sun et al., 1998; Qi et al., 2001; Lu et al., 2002). Olig2, which specifically demarcates the pMN domain and is expressed throughout oligodendrocyte (OG) development, is critical for spinal cord oligodendrogenesis since, in its absence, expression of PDGFαR, Sox10, myelin basic protein (MBP), and proteolipid protein (PLP)/DM20 are undetectable (Lu et al., 2002). Mice deficient in Pax6 exhibit a delay of 1 day and a dorsalward shift in the appearance of PDGFαR$^+$ cells (Sun et al., 1998). Nkx2.2 is important in the spatial aspect of PDGFαR$^+$ OPC specification, as demonstrated by a ventral expansion, as well as an increased number of Olig2$^+$ cells (Qi et al., 2001). It has also been hypothesized that a separate OPC lineage arises from the Nkx2.2$^+$ V3 interneuron progenitor (p3) domain. In addition, Nkx2.2 is important in OPC maturation due to the fact that Nkx2.2 mutant mice display not only a delay and reduction in MBP and PLP/DM20 expression in the white matter but also an absence of these markers in the gray matter (Qi et al., 2001).

Although numerous other TFs are expressed by cells of the OG lineage, researchers have been unable to define where they ultimately lay in the transcriptional control of oligodendrogenesis. One TF that has been shown to be expressed by O4$^+$ pro-oligodendrocytes (pro-OGs) is Hoxa2 (Hao et al., 1999). *Hoxa2* is one of

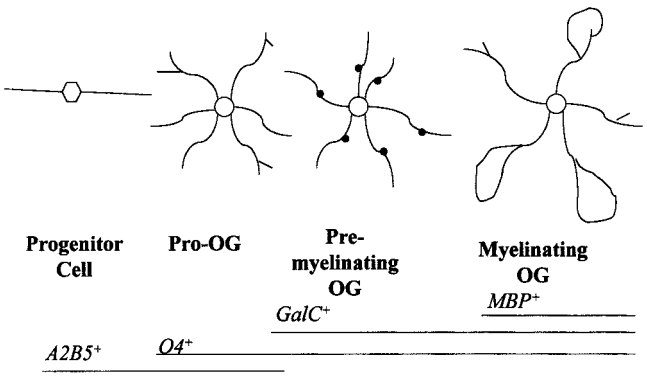

Fig. 2. Oligodendrocytes (OGs) progress through four distinct stages in vitro, characterized by unique morphological and antigenic phenotypes (Sommer and Schachner, 1981; Abney et al., 1983; Raff et al., 1983, 1985; Noble et al., 1988; Bansal et al., 1989; Fok-Seang and Miller, 1994; Asou et al., 1995; Duchala et al., 1995; reviewed in Miller, 1996).

39 mouse and human *Hox* genes that are organized into four clusters (Hox A, B, C, D) located on different chromosomes (Scott, 1992; Favier and Dollé, 1997; Santagafi and Rijla, 2003). These genes are characterized by a 180-base pair homeobox, which encodes a 60-amino acid homeodomain (McGinnis et al., 1984a,b). *Hox* genes can regulate the expression of downstream effector genes by binding to specific nucleotide sequences through the homeodomain (Hoey and Levine, 1988; Hirsch et al., 1990; Jones et al., 1992, 1993). We have further investigated Hoxa2 expression, as well as its potential role(s) in OG development. Our results suggest that although Hoxa2 is expressed by cells of the OG lineage, it does not appear to be critical for the specification or early maturation of these cells in the murine spinal cord.

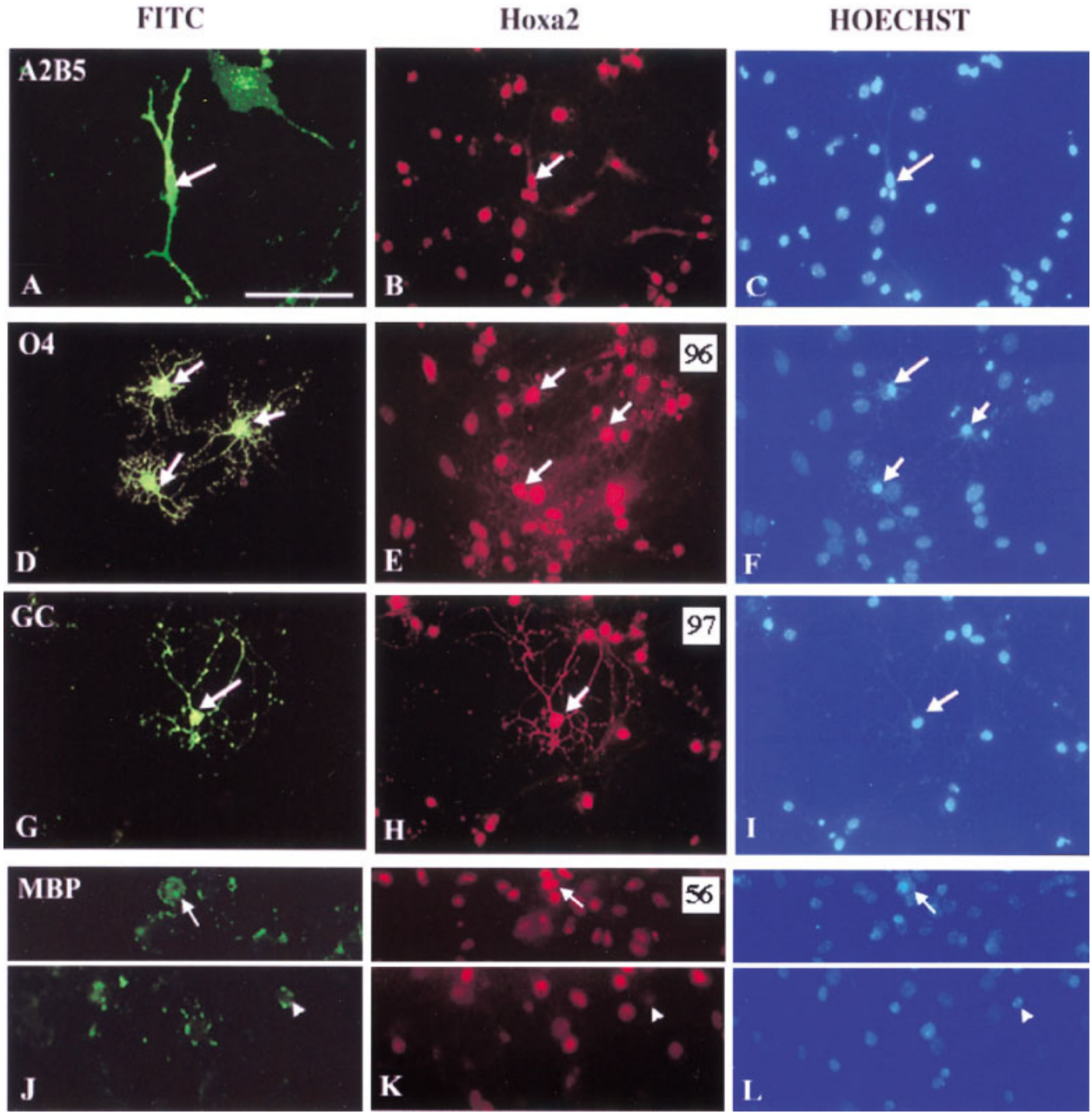

Fig. 3. Hoxa2 was expressed throughout oligodendrocyte (OG) development. Primary mixed glial cell cultures from neonatal murine cerebral hemispheres were grown in culture for 18 days. Double immunofluorescent staining was conducted with each of the four OG markers [A2B5 (**A**), O4 (**D**), GalC (**G**), and MBP (**J**)] and Hoxa2 (**B,E,H,K**). Cells were visualized using nuclear fluorescent Hoechst counterstain (**C,F,I,L**). Each row illustrates micrographs obtained from individual filters [fluorescein (A,D,G,J), rhodamine (B,E,H,K), and DAPI (C,F,I,L)] of the same field. Arrows demarcate representative Hoxa2 immunoreactive cells. Arrowheads demarcate a $Hoxa2^{-}MBP^{+}$ cell. The insets at the top right corner of the rhodamine micrographs (E,H,K) show the percentage of $O4^{+}$, $GalC^{+}$, and MBP^{+} cells that are immunoreactive for Hoxa2, respectively. [Percentages depict averages calculated from two coverslips in which 100–200 OGs were counted. The percentage of $A2B5^{+}Hoxa2^{+}$ cells was not determined because the monoclonal A2B5 antibody binds to cell-surface antigens on oligodendrocyte type 2 astrocyte (O-2A) precursor cells, which can give rise to either OGs or type 2 astrocytes depending on culture conditions (Eisenbarth et al., 1979; Abney et al., 1983; Raff et al., 1983), and therefore is not specific to the OG lineage.] Scale bar = 100 μm.

MATERIALS AND METHODS

Primary Glial Cultures and Immunocytochemistry

Cerebral hemispheres from newborn mice were dissected in Dulbecco's modified Eagle's medium/ F12/10% fetal bovine serum (DMEM/F12/10% FBS). Subsequently, single cell suspensions, obtained by forcing diced tissue through a 75-μm Nitex mesh, were plated in DMEM/F12/10% FBS for 4 days to allow adherence of the cells. They were then cultured for two 1-week periods, respectively, in a B104 conditioned medium

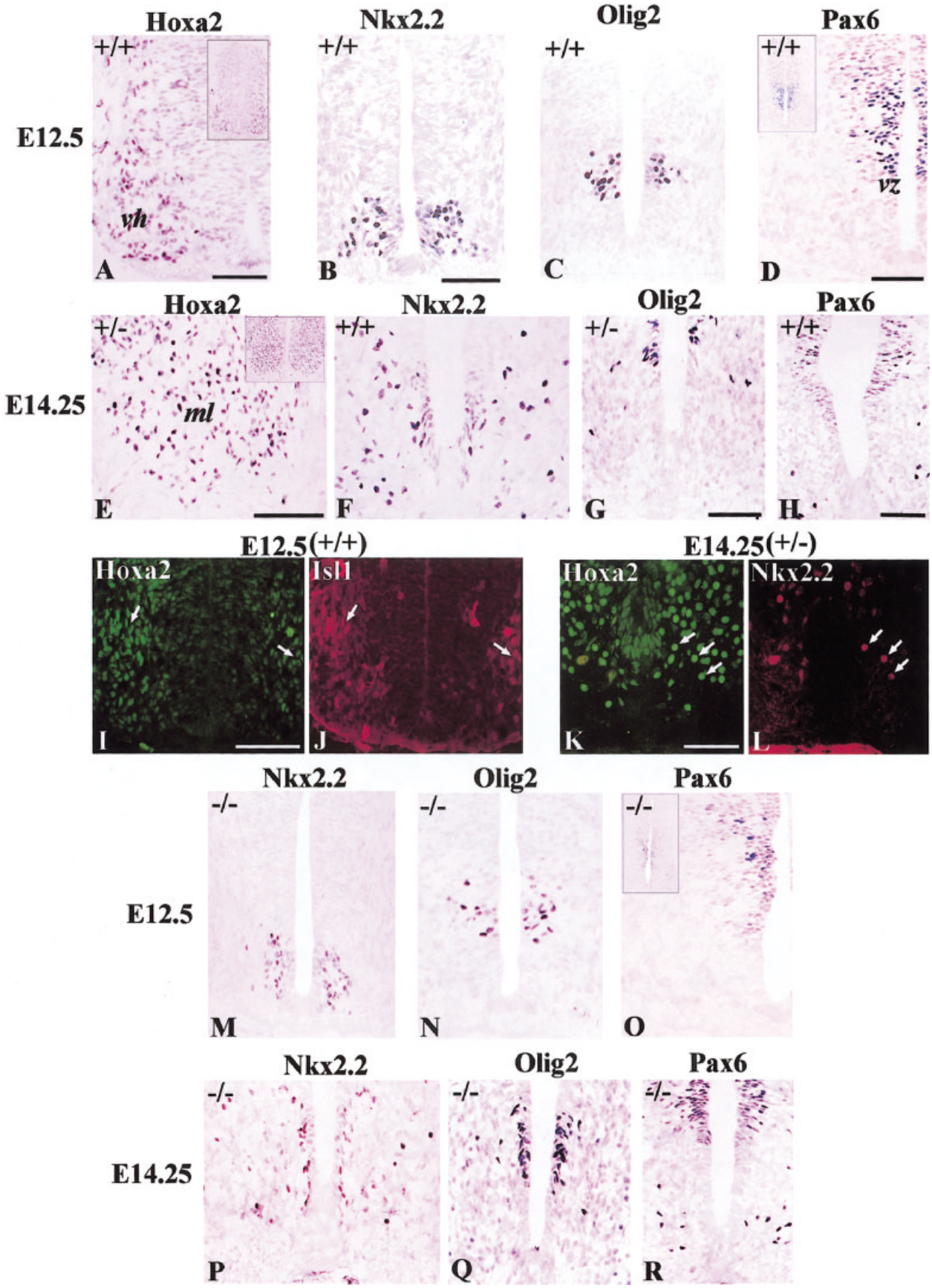
Hoxa2
Nkx2.2
Olig2
Pax6
E12.5
+/+
vh
vz
A
B
C
D
E14.25
+/-
ml
E
F
G
H
E12.5(+/+)
E14.25(+/-)
Hoxa2
Isl1
Nkx2.2
I
J
K
L
-/-
M
N
O
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Figure 4.

and a low serum-containing (0.3%) growth medium (GP medium) [described previously by Gard and Pfeiffer (1990) and Doucette and Devon (1994)] supplemented with 10 ng/ml basic fibroblast growth factor (bFGF). Culture medium was changed every 4 days.

Immunocytochemistry used the following primary antibodies: anti-Hoxa2 (1:800; Hao et al., 1999), A2B5 (1:10, hybridoma; American Type Cell Culture [ATCC]), O4 (1:10, hybridoma; Sommer and Schachner, 1981), anti-galactocerebroside (GalC) (1:20, hybridoma; Ranscht et al., 1982), and anti-MBP (1:800; Sternberger Monoclonals, Lutherville, MD). A2B5, O4, GalC, and MBP were used to mark cells at different stages of OG development (Fig. 2). In particular, the monoclonal antibody A2B5 was used to recognize a complex ganglioside expressed on the surface of OPCs (Eisenbarth et al., 1979; Abney et al., 1983). The monoclonal antibody O4 recognized surface epitopes expressed by pro-OGs (Bansal et al., 1989). GalC, the major galactosphingolipid of myelin, marked pre-myelinating OGs (Ranscht et al., 1982). Finally, expression of myelin proteins, such as MBP, marked mature myelinating OGs. Double labeling Hoxa2 with A2B5, O4, or GalC was conducted using a procedure previously described by Doucette and Devon (1994) with the following modifications: live cells were incubated for 30 min at 37°C in A2B5, O4, or anti-GalC monoclonal antibodies diluted in phosphate-buffered saline (PBS); the blocking and permeabilization steps were combined with a 30-min incubation in 3% skim milk powder solution containing 0.1% Triton X (3% SM/0.1% TX); and cells were incubated for 4 h at room temperature (RT) in anti-Hoxa2 diluted in 1% SM/0.03% TX. The secondary antibodies used included goat anti-mouse IgM fluorescein isothiocyanate (FITC) (1:100, A2B5/O4; Sigma, Oakville, ON), donkey anti-mouse IgG FITC (1:50, GalC; BIO/CAN Scientific, Mississauga, ON), and goat anti-rabbit IgG CY3 (1:200, Hoxa2; BIO/CAN Scientific). Between incubations, cultures were washed twice for 5 min in PBS. Double immunostaining Hoxa2 with MBP was done using a procedure previously described by Doucette and Devon (1994), with two modifications: (1) cells were incubated in anti-MBP and then anti-Hoxa2 for 30 min and 4 h, respectively; (2) between anti-MBP and anti-Hoxa2 incubations, cells were rinsed in PBS and were then incubated for 30 min in 3% SM/0.1% TX. The nuclei in all cell cultures were stained using Hoechst dye (Sigma, ON). The percentage of OGs ($O4^+$, $GalC^+$, and MBP^+ cells) that expressed Hoxa2 was calculated from 2 coverslips in which 100–200 OGs were counted.

Hoxa2 Transgenic Mice and Immunohistochemistry

Hoxa2$^{+/+}$, *Hoxa2*$^{+/-}$, and *Hoxa2*$^{-/-}$ C57 black mice (Gendron-Maguire et al., 1993) were obtained by timed heterozygous matings. They were staged according to the Carnegie (Butler and Juurlink, 1987) and Theiler (1972) staging systems. The embryonic ages (E) and corresponding Carnegie (C)/Theiler (T) stages used were as follows: E12.5 (C16/T19), E14.25 (C19+/T21), E16 (C23/T23), and E18 (T25); the Carnegie staging system does not go beyond stage 23. Although *Hoxa2*$^{-/-}$ mice could be identified by pinna deformities at later stages of embryonic development (i.e., E14+), genotypes were confirmed by polymerase chain reaction (PCR) analysis (Gendron-Maguire et al., 1993).

Mouse embryos were fixed by immersion in 4% paraformaldehyde at 4°C for 24–48 h depending on their age. Embryos were then rinsed and stored in 20% sucrose until they were processed. Serial cryostat sections (8 μm) were collected on gelatin-coated coverslips and allowed to dry for ~1 h prior to staining. The following primary antibodies were used: anti-Olig2 [gift of Dr. Hirohide Takebayashi (Takebayashi et al., 2000); E12.5, 1:4,000; rest, 1:2,000], anti-Nkx2.2 (hybridoma; Developmental Studies Hybridoma Bank; E12.5, 1:4; rest, 1:2), anti-Hoxa2 (Hao et al., 1999; E12.5, 1:3,000; rest, 1:1,500), and anti-Pax6 (Developmental Studies Hybridoma Bank; E12.5, 1:1,000; rest, 1:500). Immunohistochemical analysis of Hoxa2 and Olig2 was performed with a procedure previously described by Hao et al. (1999). The following modifications were made with regard to time intervals: PBS washes were 8 min instead of 10 min; 3% SM/0.1% TX blocks/permeabilizations were 20 min versus 30–60 min; and incubation in the secondary antibody was 30 min versus 45 min.

Immunohistochemical analysis of Nkx2.2 and Pax6 used a modified Mouse on Mouse (MOM)™ (Vector Laboratories, Burlingame, CA) staining procedure. Briefly, sections were washed twice in PBS for 8 min, followed by a 20-min permeabilizing/blocking period in

Fig. 4. Transcription factor (TF) expression during oligodendrocyte precursor cell (OPC) specification in the spinal cords of *Hoxa2*$^{+/+}$, *Hoxa2*$^{+/-}$, and Hoxa2$^{-/-}$ mice. Top two rows: Transverse thoracic spinal cord sections from E12.5 (**A–D**) and E14.25 (**E–H**) Hoxa2$^{+/+}$ and *Hoxa2*$^{+/-}$ mice were immunolabeled with anti-Hoxa2 (A,E) anti-Nkx2.2 (B,F), anti-Olig2 (C,G), and anti-Pax6 (D,H) as described in Materials and Methods. Initially Hoxa2 expression was localized to the ventral horns (*vh*) at E12.5 (A), after which it was expressed throughout the mantle layer (*ml*) (E). Nkx2.2$^+$ and Olig2$^+$ cells, which were initially localized to distinct domains in the vVZ at E12.5 (B,C), had begun to migrate into the *ml* by E14.25 (F,G). Pax6 was expressed in the ventricular zone (*vz*) at both stages (D,H), with occasional immunoreactive cells found in the *ml* by E14.25 (H). Middle row: Transverse thoracic spinal cord sections from E12.5 (**I,J**) and E14.25 (**K,L**) *Hoxa2*$^{+/+}$ and *Hoxa2*$^{+/-}$ mice were double immunolabeled with anti-Hoxa2 (I,K) and anti-Islet1(Isl1)(J) or anti-Nkx2.2(L), respectively. Each fluorescent image illustrates micrographs obtained from individual filters [fluorescein (I,K) and rhodamine (J,L)] of the same field. Hoxa2 was co-expressed by Isl1$^+$ motor neurons at E12.5 (I,J). At E14.25 Hoxa2$^+$Nkx2.2$^+$ cells were demonstrated in the mantle layer (K,L). Bottom two rows: Transverse thoracic spinal cord sections from E12.5 (**M–O**) and E14.25 (**P–R**) *Hoxa2*$^{-/-}$ mice were immunolabeled with anti-Nkx2.2 (M,P), anti-Olig2 (N,Q), and anti-Pax6 (O,R). Expression profiles of Nkx2.2, Olig2, and Pax6 were similar in the presence (B–D,F–H) and absence (M–R) of *Hoxa2*. Analyses were conducted with at least three spinal cord sections at each stage of development in both the presence and the absence of *Hoxa2*. Significant discrepancies were not observed and any inconsistencies were primarily due to the way the sections were cut, in particular, the location (G,Q) and shape (H,R) of the central canal varied between some sections. Scale bars = 100 μm.

3% SM/0.1% TX. After two 2-min washes in PBS, sections were incubated for 1 h in MOM Mouse Ig blocking solution. Following a 5-min block in MOM diluent, sections were incubated at RT for 30 min and 10 min in primary and secondary antibodies, respectively. Two 2-min PBS washes were done between incubations. Endogenous peroxidase activity was then blocked by an 8-min incubation in a 0.3% hydrogen peroxide solution. After two 4-min PBS washes, sections were incubated in avidin-biotin complex (Vectastain® Elite ABC; Vector) for 20 min at RT. The sections were subsequently washed for 8 min in PBS and then 0.175 M sodium acetate, respectively. For diaminobenzidine tetrahydrochloride (DAB) staining, a 2-min incubation was performed as described previously by Hao et al. (1999). Sections were then washed in PBS, dehydrated, and mounted in Permount® (Fisher Scientific, Nepean, ON).

For immunofluorescent double labeling, sections were washed twice in PBS for 8 min, followed by a 20-min block/permeabilization in 3% SM/0.1% TX. They were then incubated overnight in primary antibodies [anti-Hoxa2 (1:200), anti-Olig2 (1:2,000), anti-Pax6 (1:100), anti-Nkx2.2 (concentrate), anti-Islet1 (Developmental Studies Hybridoma Bank, 1:3,000), and anti-β-tubulin (1:8,000)] diluted in 1% SM/0.03% TX. After two 8-min PBS washes, sections were incubated for 3 h in Alexa 594 and 488 secondary antibodies (Molecular Probes, Eugene, OR). Finally, sections were rinsed in PBS and were mounted in Citifluor® (Merivac, Montreal, QC). (When sections were stained for β-tubulin an additional 8-min incubation in Hoechst dye was performed to identify nuclei.)

RESULTS

Hoxa2 Is Expressed Throughout OG Development In Vitro

Immunocytochemical analysis of primary mixed neopallial glial cell cultures demonstrated that Hoxa2 was expressed throughout OG development, diminishing only with the acquisition of a myelinating phenotype (Fig. 3). In particular, greater than 95% of $O4^+$ pro-OGs and $GalC^+$ pre-myelinating OGs expressed Hoxa2 (Fig. 3E,H). As OGs matured to the MBP^+ myelinating phenotype, the percentage of Hoxa2-expressing cells dropped to 56% (Fig. 3K). The location of immunoreactivity was primarily nuclear; however, at the pre-myelinating stage cytoplasmic staining was also observed. Although Hoxa2 was expressed in most cells in the OG lineage, its expression in these mixed glial cell cultures was not limited to OGs. This was demonstrated by the expression of Hoxa2 in cells that were A2B5, O4, GalC, and MBP-negative (Fig. 3B,E,H,K).

Hoxa2 Expression Is Not Required for OPC Specification in the Murine Spinal Cord

At E12.5 (C16/T19) Hoxa2 was expressed predominantly in the ventral horns (Fig. 4A). In contrast, Nkx2.2 and Olig2, which may mark separate OPC lineages, were expressed in distinct regions of the vVZ in $Hoxa2^{+/+}$ and $Hoxa2^{+/-}$ mice (Fig. 4B,C; data not shown). In particular, Nkx2.2 was expressed in the p3 domain, which is adjacent to the floorplate. Olig2 was expressed just dorsal to the $Nkx2.2^+$ p3 domain in the pMN domain. Although Olig2 expression was restricted to the vVZ, some $Nkx2.2^+$ cells were found in the adjacent mantle layer (Fig. 4B). Double immunofluorescent staining demonstrated that some of the $Nkx2.2^+$ cells located in the mantle layer co-expressed Hoxa2 (data not shown). Many $Islet1^+$ motor neurons in the mantle layer also co-expressed Hoxa2 (Fig. 4I,J).

At E14.25 (C19+/T21), Hoxa2 was expressed throughout the mantle layer (Fig. 4E). Although at E14.25, Nkx2.2 and Olig2 continued to be expressed in the vVZ, $Nkx2.2^+$ and $Olig2^+$ cells were also found in the mantle layer (Fig. 4F,G). Occasionally, Olig2-immunoreactive cells were observed in the marginal layer at this stage of development (data not shown). Double immunofluorescent staining demonstrated that few if any cells exhibited co-expression of Nkx2.2 and Olig2 at E14.25 (C19+/T21) (data not shown). However, most $Nkx2.2^+$ cells located in the mantle layer expressed Hoxa2 (Fig. 4K,L).

The absence of *Hoxa2* expression in the vVZ during OPC specification would suggest that it may not be critical for this process in the spinal cord. To test this directly, immunohistochemical analysis of $Hoxa2^{+/+}$, $Hoxa2^{+/-}$, and $Hoxa2^{-/-}$ spinal cord sections were conducted to determine whether Nkx2.2, Pax6, and/or Olig2 expression profiles were altered in the absence of *Hoxa2*. $Hoxa2^{+/+}$ and $Hoxa2^{+/-}$ mice were used interchangeably to represent the presence of *Hoxa2*, since expression profiles were similar in both genotypes. Single immunohistochemical analysis of $Hoxa2^{+/+}$ mice demonstrated that at E12.5 (C16/T19) Olig2 was expressed in an adjacent nonoverlapping domain with Nkx2.2, whereas Pax6 was expressed dorsal to the $Nkx2.2^+$ domain (Fig. 4B–D). Comparison of these expression profiles with those obtained from $Hoxa2^{-/-}$ mice demonstrated that they were maintained in the absence of *Hoxa2* (4M-O). At E14.25 (C19+/T21), when immunoreactive cells were still evident in the vVZ, expression profiles of Nkx2.2, Olig2, and Pax6 were similar regardless of genotype (Fig. 4F–H,P–R). Double immunofluorescent staining of Nkx2.2 and Olig2 or Pax6 at E12.5 (C16/T19) confirmed that the expression profiles were maintained in the absence of *Hoxa2* (Fig. 5).

Hoxa2 Is Not Required for Early Maturation of OGs in the Murine Spinal Cord

At E16 (C23/T23) and E18 (T25), Hoxa2 was expressed throughout the mantle layer, with occasional immunoreactive cells also observed in the marginal layer (Fig. 6A,E,I–K). $Nkx2.2^+$ and $Olig2^+$ cells continued to migrate into the mantle layer, and eventually

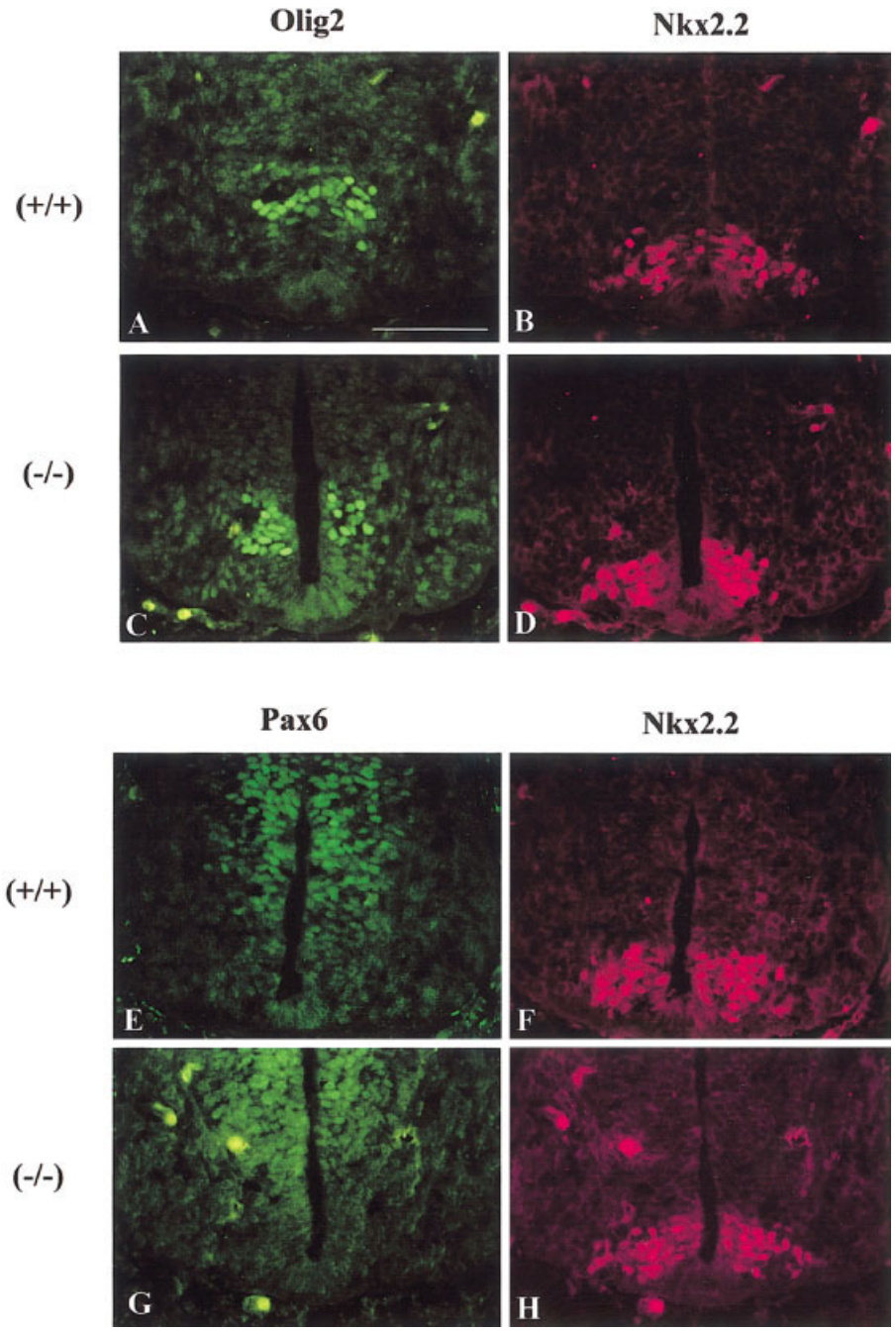
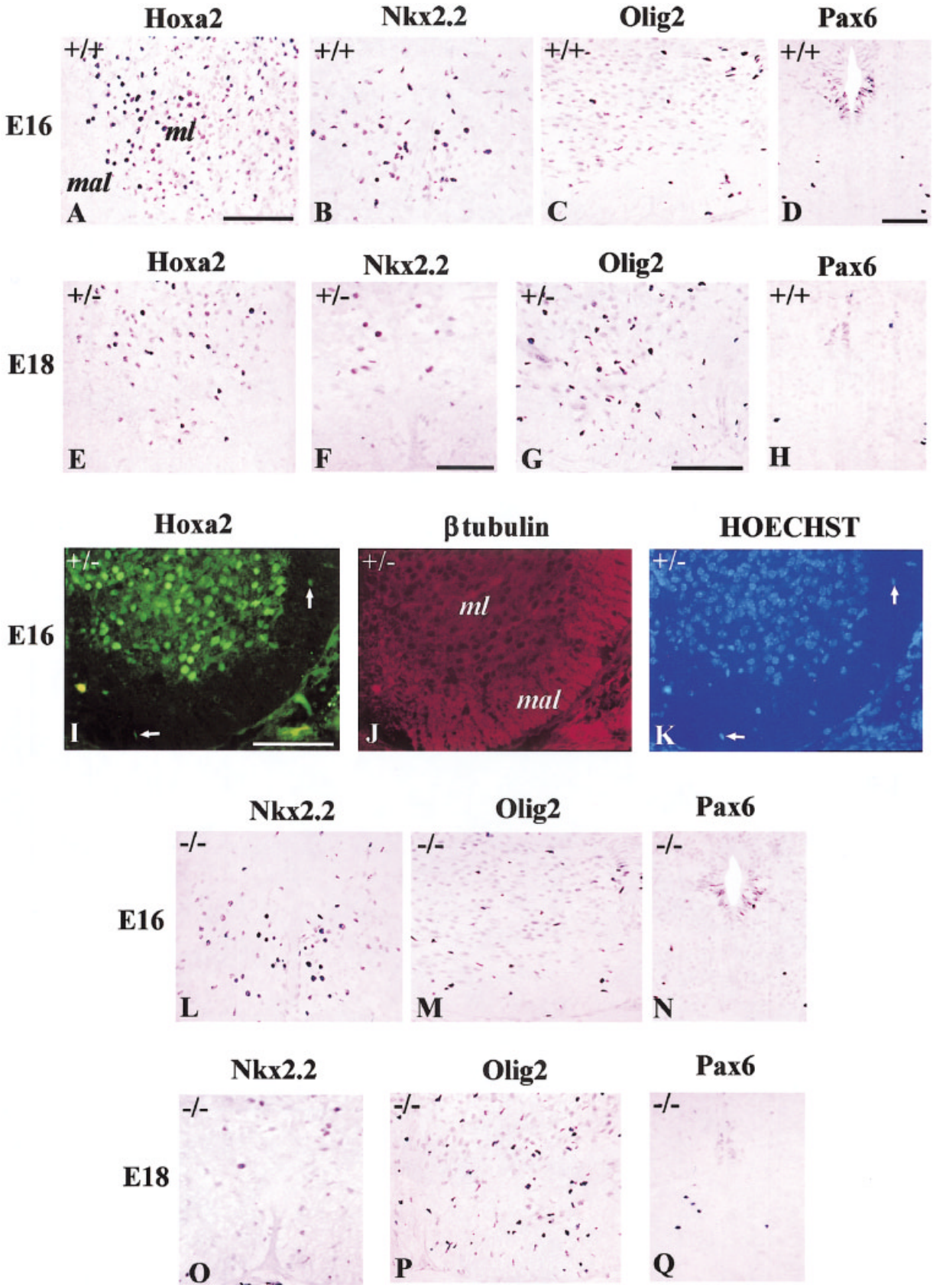


Fig. 5. Oligodendrocyte precursor cell (OPC) specification occurred normally in the absence of *Hoxa2*. Transverse thoracic E12.5 *Hoxa2*$^{+/+}$ and *Hoxa2*$^{-/-}$ spinal cord sections were subjected to double immunofluorescent labeling with anti-Nkx2.2 and anti-Olig2 (**A–D**) or anti-Nkx2.2 and anti-Pax6 (**E–H**) antibodies as described in Materials and Methods. Each row illustrates micrographs obtained from individual filters [fluorescein (A,C,E,G) and rhodamine (B,D,F,H)] of the same field. Nkx2.2 and Olig2 exhibit adjacent nonoverlapping expression domains in both wild-type (A,B) and mutant (C,D) mice. Nkx2.2 and Pax6 exhibit similar expression domains in both wild-type (E,F) and mutant (G,H) mice. Scale bar = 100 μm.

these cells began to accumulate in the marginal layer of *Hoxa2*$^{+/+}$ and *Hoxa2*$^{+/-}$ mice. This migration was accompanied by a reduction and the eventual disappearance of Nkx2.2^{+} and Olig2^{+} immunoreactive cells in the vVZ at E16 (C23/T23) and E18 (T25), respectively (Fig. 6B,C,F,G). Comparisons of Nkx2.2 and Olig2 expression profiles from *Hoxa2*$^{+/+}$ and *Hoxa2*$^{+/-}$ mice with that of *Hoxa2*$^{-/-}$ mice failed to reveal any consistent changes in expression profiles (Fig. 6B–D,F–H,L–Q).

Double labeling immunohistochemical analysis of Olig2 and Nkx2.2 expression in *Hoxa2*$^{+/+}$ and *Hoxa2*$^{+/-}$ mice demonstrated that with continued maturation co-expression of these TFs became evident, especially in the marginal layer (Fig. 7A,B). Comparison of these co-expression profiles with those obtained from *Hoxa2*$^{-/-}$ mice showed no detectable alterations (Fig. 7C,D). In addition, comparisons of *Hoxa2*$^{+/+}$, *Hoxa2*$^{+/-}$, and *Hoxa2*$^{-/-}$ mice demonstrated that the accumulation of Olig2^{+} cells in the marginal layer of the developing spinal cord proceeded similarly regardless of genotype (Fig. 7E–H). Thus, the early maturation of OGs proceeded normally in the absence of the *Hoxa2* gene.


Hoxa2
Nkx2.2
Olig2
Pax6
E16
+/+
ml
mal
A
B
C
D
E18
+/-
E
F
G
H
βtubulin
HOECHST
I
J
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-/-
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Figure 6.

DISCUSSION

OG development in vitro progresses through a number of distinct stages, which are characterized by specific antigenic phenotypes (Fig. 2). During the early stages of oligodendrogenesis, OPCs and pro-OGs, which are characterized by A2B5 and/or O4 staining, actively proliferate and migrate. Subsequently, loss of these traits and the emergence of GalC expression signal terminal differentiation into pre-myelinating OGs. Finally, progression to the mature myelinating phenotype occurs with the production of myelin proteins and the elaboration of sheet-like membranes (Abney et al., 1983; Fok-Seang and Miller, 1994; Duchala et al., 1995; reviewed in Raff et al., 1985; Noble et al., 1988; Miller, 1996).

Although in situ hybridization and immunohistochemical analysis have shown that *Hoxa2* is expressed in regions of the central nervous system (CNS), including the myelencephalon, diencephalon, and spinal cord (Tan et al., 1992; Wolf et al., 2001), Hao et al. (1999) were the first to demonstrate the expression of Hoxa2 by cells of the OG lineage. In particular, Hao et al. (1999) demonstrated that $O4^+$ pro-OGs, obtained from E15 murine cerebral hemispheres, expressed Hoxa2. To confirm these findings, as well as further investigate Hoxa2 expression in cells of the OG lineage, an immunocytochemical analysis was performed on primary mixed glial cultures obtained from the cerebral hemispheres of newborn mice. These cultures consisted of oligodendroglia that developed on top of an astrocytic monolayer. The results showed that although Hoxa2 was expressed throughout OG development (Fig. 3), the location of immunoreactivity, as well as the number of $Hoxa2^+$ OGs changed with maturation. In particular, Hoxa2 expression by OGs was primarily nuclear (Fig. 3B,E), however, cytoplasmic staining was also observed at the pre-myelinating stage (Fig. 3H). The reason for this cytoplasmic staining is unknown, but it has been demonstrated with several other TFs (Armstrong et al., 1995; Wang et al., 2001). Subsequently, there was a reduction in the number of $Hoxa2^+$ myelinating OGs in comparison to $Hoxa2^+$ pre-myelinating OGs (Fig. 3H,K).

Researchers recently identified a subfamily of basic helix-loop-helix (bHLH) TFs known as the Olig genes, whose members include Olig1, Olig2, and Olig3 (Lu et al., 2000; Takebayashi et al., 2000; Zhou et al., 2000). Olig1 and Olig2 are co-expressed by cells in the pMN domain of the developing spinal cord prior to PDGFαR (Lu et al., 2000; Zhou et al., 2000). In contrast, Olig3, which is transiently expressed in the embryonic CNS, is not co-expressed with Olig2 (Takebayashi et al., 2002). $Olig1^+$ and $Olig2^+$ cells disperse throughout the spinal cord in a ventral to dorsal manner similar to that of $PDGF\alpha R^+$ cells (Zhou et al., 2000). Furthermore, double in situ hybridization histochemical analysis has also demonstrated that $PDGF\alpha R^+$ cells co-express Olig1 and Olig2 (Lu et al., 2000; Zhou et al., 2000). Hence they are believed to be the earliest known markers of the OG lineage. In addition, Olig1 and Olig2 continue to be expressed in OGs into adulthood (Lu et al., 2000; Takebayashi et al., 2000).

In the present study, Olig2 was used to identify cells of the $PDGF\alpha R^+$ OPC lineage. Although Olig2 initially specifies motor neuron precursors at the early stages of spinal cord development, it is downregulated as the cells differentiate (Novitch et al., 2001). In addition, it is not expressed by glial fibrillary acidic protein $(GFAP)^+$ astrocytes (Takebayashi et al., 2000), which are also found in the CNS. These findings suggest that during periods of oligodendrogenesis in the spinal cord, Olig2 is specific to the OG lineage. Olig2 was used as our OG marker instead of Olig1 for two reasons. First, whereas the number of $Olig2^+$ cells has been shown to be similar to that of $PDGF\alpha R^+$ cells, $Olig1^+$ cells appeared to outnumber $PDGF\alpha R^+$ cells (Zhou et al., 2000). Second, Olig2 has been shown to be critical for OG development in the spinal cord since OPCs and mature OGs failed to develop in its absence. In contrast, $PDGF\alpha R^+$ cells appeared on schedule in the absence of Olig1; however, there was a delay in the appearance of Sox10, MBP, and PLP/DM20 expression in the white matter of the spinal cord (Lu et al., 2002).

Research on OG development in the chick has led to the hypothesis that a separate OPC lineage may also arise from the $Nkx2.2^+$ p3 domain. Although all migrator $Olig2^+$ cells co-express Nkx2.2 in the chick spinal cord, $Nkx2.2^+$ cells that are weak or negative for Olig2 expression were found, and a portion of these cells expressed O4 (Zhou et al., 2001; Fu et al., 2002). In addition, ~33% of $A2B5^+$ glial restricted precursor cells, which were immunopurified from ~E13.5 rat spinal cord, have been shown to be immunoreactive for Nkx2.2. These $Nkx2.2^+$ cells were believed to be OGs because few β-$tubulin^+$ neurons and $GFAP^+$ astrocytes expressed Nkx2.2 (Qi et al., 2001).

In our study, immunohistochemical analyses were initiated at E12.5 (C16/T19) since Olig2 expression

Fig. 6. Transcription factor (TF) expression during oligodendrocyte precursor cell (OPC) maturation in the spinal cords of $Hoxa2^{+/+}$, $Hoxa2^{+/-}$, and $Hoxa2^{-/-}$ mice. Top two rows: Transverse thoracic spinal cord sections from E16 (**A–D**) and E18 (**E–H**) $Hoxa2^{+/+}$ and $Hoxa2^{+/-}$ mice were immunolabeled with anti-Hoxa2 (A,E) anti-Nkx2.2 (B,F), anti-Olig2 (C,G), and anti-Pax6 (D,H) as described in Materials and Methods. At these stages Hoxa2 was expressed predominantly in the mantle layer (*ml*) (A,E). $Nkx2.2^+$ and $Olig2^+$ cells continued to migrate into the *ml* and eventually accumulated in the marginal layer (*mal*) (B,C,F,G). Pax6 expression was concentrated in the ventricular zone at these stages, with occasional immunoreactive cells found in the *ml* (D,H). Middle row: Transverse thoracic spinal cord section from an E16 (**I–K**) $Hoxa2^{+/-}$ mouse was double immunolabeled with anti-Hoxa2 (I) and anti-βtubulin (J). β-Tubulin staining was used to demarcate the *mal*, which is populated primarily by glial cells. Cells were visualized using nuclear fluorescent Hoechst counterstain (K). Each fluorescent image illustrates micrographs obtained from individual filters [fluorescein (I), rhodamine (J), and DAPI (K)] of the same field. At later stages of development occasional $Hoxa2^+$ cells (arrows) were found in the glial-rich *mal*. Bottom two rows: Transverse thoracic spinal cord sections from E16 (**L–N**) and E18 (**O–Q**) $Hoxa2^{-/-}$ mice were immunolabeled with anti-Nkx2.2 (L,O), anti-Olig2 (M,P), and anti-Pax6 (N,Q). Expression profiles of Nkx2.2, Olig2, and Pax6 were similar in both the presence (B–D,F–H) and absence (L–Q) of Hoxa2. Scale bars = 100 μm.

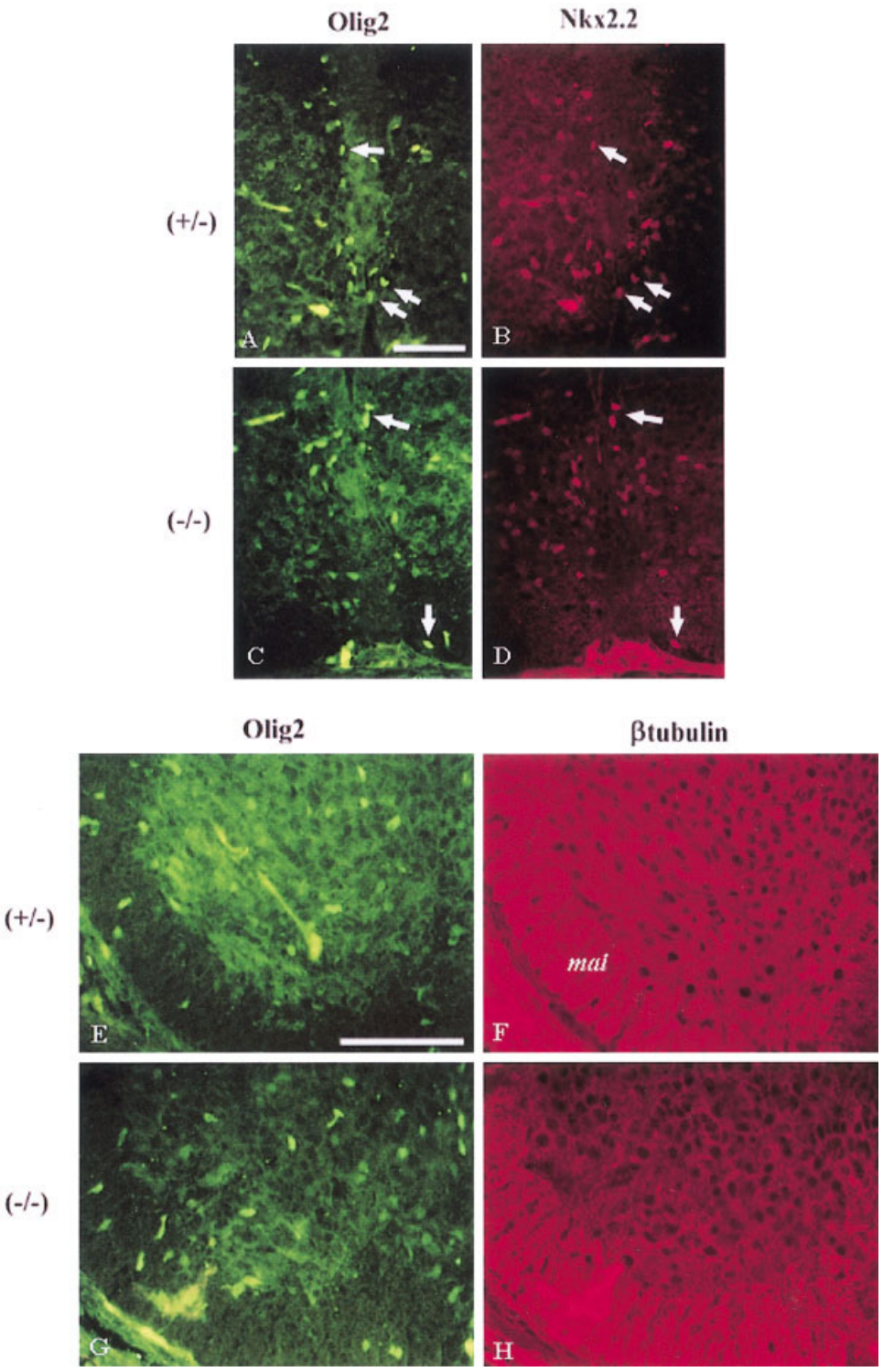

Fig. 7. Early oligodendrocyte (OG) maturation proceeded normally in the absence of *Hoxa2*. Transverse thoracic E16 Hoxa2$^{+/-}$ and *Hoxa2*$^{-/-}$ spinal cord sections were subjected to double immunofluorescent labeling with anti-Nkx2.2 and anti-Olig2 (**A–D**) or anti-Olig2 and anti-β-tubulin (**E–H**) as described in Materials and Methods. Each row illustrates micrographs obtained from individual filters [fluorescein (A,C,E,G), rhodamine (B,D,F,H)] of the same field. With continued maturation, cells began to co-express Olig2 and Nkx2.2 (arrows) in both heterozygous (A,B) and mutant (C,D) mice. Furthermore, the migratory pattern of Olig2^{+} cells was similar in both heterozygous (E,F) and mutant (G,H) mice, as demonstrated by the accumulation of immunoreactive cells in the marginal layer (*mal*). Scale bar = 100 μm.

would be downregulated in postmitotic motor neurons at this time. In addition it corresponds to the emergence of PDGFαR expression in the vVZ (Sun et al., 1998). At this stage, Hoxa2 was expressed predominantly in the ventral horns (Fig. 4A), which was in direct contrast to Nkx2.2 and Olig2, which were expressed in the vVZ (Fig. 4B,C) of *Hoxa2*$^{+/+}$ and *Hoxa2*$^{+/-}$ mice. Hence, our results suggest that Hoxa2 expression in OPCs occurs following specification. At later stages, overlapping expression profiles of Hoxa2 with Nkx2.2 and Olig2 in the mantle layer suggest that Hoxa2 is expressed by migratory OG cells. Unfortunately, we were unable to conclusively show double labeling of Hoxa2 and Olig2 because both antibodies

were rabbit polyclonals. However, we were able to demonstrate co-expression of Hoxa2 by Nkx2.2$^+$ cells (Fig. 4K,L), which have been hypothesized to represent a separate population of OPCs (Fu et al., 2002) as well as co-expression of Nkx2.2 and Olig2 (Fig. 7A–D).

With continued maturation Hoxa2 expression in OGs appeared to diminish as suggested by the finding that Nkx2.2$^+$ and Olig2$^+$ cells greatly outnumbered Hoxa2$^+$ cells in the marginal layer (Fig. 6A–C,E–G). In contrast, Hoxa2$^+$ cells outnumbered Nkx2.2$^+$ and Olig2$^+$ cells in the mantle layer, which supported the in vitro finding that cells in addition to OGs also express Hoxa2 (Fig. 6A–C,E–G). For example, Hoxa2 has been shown to be expressed by Islet1$^+$ motor neurons and GFAP$^+$ astrocytes (Hao et al., 1999) (Fig. 4I,J). Although Hoxa2 is not specific to the OG lineage, no TFs have been shown to be expressed exclusively by OGs.

As most research regarding OG development has involved the analysis of the rodent spinal cord, we decided to focus our study on this area of the CNS. Although Shh dependence and the ventral origin of OPCs are similar in the spinal cord and brain (Pringle and Richardson, 1993; Ono et al., 1995; Hall et al., 1996; Orentas et al., 1999; Tekki-Kessaris et al., 2001; Alberta et al., 2001; reviewed in Woodruff et al., 2001), differences in OG development in these regions have emerged. For example, Nkx2.2$^+$Olig2$^+$ cells are found in the vVZ of the chick spinal cord prior to emigration (Zhou et al., 2001; Fu et al., 2002). In contrast, in the chick hindbrain only Nkx2.2$^+$ cells are found in the vVZ at the onset of oligodendrogenesis with Olig2 expression only evident in migratory cells (Fu et al., 2002). Takebayashi et al. (2000) have also shown discrepancies in Olig1 and Olig2 expression in the murine embryonic brain versus spinal cord. In particular, in the murine embryonic brain Olig2 displayed a broader expression domain than Olig1. In addition it was shown that in certain brain regions numerous Olig2$^+$ cells were found, few of which co-expressed Olig1 or 2′-3′ cyclic nucleotide-3′-phosphohydrolase (CNP) (Takebayashi et al., 2000), the latter being a myelinating glial cell specific enzyme. Takebayashi et al. (2000) concluded that Olig2 may not be limited to OGs in the brain and hence may play a role in this region distinct from Olig1. This conclusion is supported by the study of Lu et al. (2002), who found that in the hindbrain and areas of the forebrain, the early stages of OG development proceeded largely unaltered in the absence of Olig2. Hence, Olig2 does not appear to play a critical role in OG development in certain brain regions. These discrepancies may be partially due to differing expression profiles of regulatory TFs by cells of the OG lineage along the anterior-posterior axis, such as the *Hox* genes.

One potential role for *Hoxa2* in OG development may be via its transcriptional regulation of neural cell adhesion molecule (NCAM). NCAM, a cell surface glycoprotein, exists as three prominent protein isoforms (120,140,180 kD), which are encoded by four predominant transcripts arising from alternative splicing and polyadenylation sites of a single gene (Barbas et al., 1988). Examination of the 5′ end of the NCAM gene has revealed homeodomain binding sites (HBSs) that have the potential to be regulated by homeobox genes (Hirsch et al., 1990; Jones et al., 1992, 1993). *Hoxc6* (*Hox3.3*) and *Hoxb9* (*Hox2.5*) have previously been shown to enhance chloramphenicol acetyltransferase (CAT) activity significantly via NCAM HBS (Jones et al., 1992, 1993). In contrast, *Hoxb8* (*Hox2.4*) decreased HBS-I$^+$/ HBS-II$^+$- directed CAT activity, as well as *Hoxb9*-enhanced activity (Jones et al., 1992). Hence NCAM promoter activity may be the result of the combined actions of numerous *Hox* genes. Furthermore, mutations in NCAM HBSs resulted in alterations in β-galactosidase reporter gene expression patterns in the embryonic spinal cord in vivo (Wang et al., 1996).

NCAMs undergo a variety of posttranslational modifications, including glycosylation by enzymes such as sialyltransferase (Rothbard et al., 1982; Finne et al., 1983; Breen et al., 1987; Breen and Regan, 1988); the activity of this enzyme is developmentally regulated (Breen et al., 1987; Breen and Regan, 1988). As a result, NCAMs vary in their sialic acid content, with the highest evident in the embryonic polysialylated form of NCAM (PSA-NCAM) (Breen et al., 1987; Breen and Regan, 1988; Rothbard et al., 1982; Finne et al., 1983). Sialic acid content has been demonstrated to be inversely related to NCAM homophilic binding properties, such as adhesion and rate of aggregation (Hoffman and Edelman, 1983; Sadoul et al., 1983). Hence *Hoxa2* could potentially enhance or repress transcription of the *NCAM* gene, which would subsequently give rise to embryonic or adult forms, depending on the activity of sialyltransferase. The expression of either the embryonic or adult form of NCAM by oligodendroglial cells may affect their motility and maturation (Wang et al., 1994; Gard et al., 1996).

Comparison of Nkx2.2, Olig2, and Pax6 expression profiles obtained from at least three spinal cord sections of E12.5, E14.25, E16, and E18 mice in both the presence (*Hoxa2*$^{+/+}$ and *Hoxa2*$^{+/-}$), and the absence of *Hoxa2* (*Hoxa2*$^{-/-}$) failed to reveal major differences. These results suggest that Hoxa2 does not appear to be critical for the OPC specification and early maturation in the murine spinal cord. One potential reason for this could be that other *Hox* genes may compensate for the loss of *Hoxa2* in the developing spinal cord. Analyzing OG development in the more anterior regions of the CNS in *Hoxa2*$^{-/-}$ mice, where *Hox* gene compensation is less likely, should demonstrate whether discrepancies in early stages of OG development exist in the absence of the *Hoxa2* gene.

The regulatory mechanisms controlling oligodendrogenesis, once elucidated, will guide the development of therapeutic approaches to treating demyelinating diseases such as multiple sclerosis (MS). MS is an inflammatory disease of the CNS characterized by focal areas of demyelination that appear to be immune driven (Prineas et al., 1993a,b; Ozawa et al., 1994; Raine, 1997a,b; Storch et al., 1998; Lucchinetti et al., 1999).

The OG progenitors are the most motile of the OG lineage cells (Gansmuller et al., 1991), which are recruited to areas of demyelination over only a very limited area (Franklin and Blakemore, 1997). There are three potential sources of remyelinating OGs: adult progenitors endogenous to or adjacent to the lesion, or mature OGs that have survived the demyelinating event (Levine et al., 2001). Remyelination, which is characterized by axons exhibiting thin myelin sheaths, fails with disease progression (Prineas et al., 1989, 1993a,1993b; Raine and Wu, 1993; Brück et al., 1994; Ozawa et al., 1994). The reason that remyelination fails remains unknown, but it may include errors in migration or maturation. PSA-NCAM is one of several molecules that appear to be important for the migratory ability of OG progenitor cells (Barral-Moran et al., 2003). Hence, elucidation of the transcriptional control by *Hox* genes of molecules involved in these processes, such as NCAMs, will help determine ways to enhance remyelination.

ACKNOWLEDGMENTS

The work was supported by a research grant from the Canadian Institutes of Health Research (to A.J.N. and J.R.D.). The authors are grateful to the Multiple Sclerosis Society of Canada for support of the technical personnel and to Ms. Sheng Yu for technical assistance with the primary cell culture preparations. D.J.N. is supported by an Rx&D/CIHR scholarship. We extend our gratitude to Dr. Hirohide Takebayashi for the Olig2 antibody. The anti-Nkx2.2 hybridoma, islet-1 monoclonal antibody developed by Dr. T.M. Jessell and the anti-Pax6 monoclonal antibody developed by Dr. A. Kawakami were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

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Cellular and Molecular Neurobiology, Vol. 24, No. 3, June 2004 (© 2004)

Hoxb4 in Oligodendrogenesis

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Received June 16, 2003; accepted August 8, 2003

SUMMARY

1. Although recent advances have provided insight into the transcriptional control of oligodendrocyte (OG) development, little information exists on the role of clustered *Hox* genes in this process. The aim of this study was to examine the expression profile of Hoxb4 in the oligodendroglial lineage.

2. Immunocytochemical analysis of primary mixed glial cultures demonstrated that Hoxb4 was expressed throughout OG development, being coexpressed with oligodendroglial markers, A2B5, O4 (97%), GalC (91%), and MBP (93%).

3. Immunohistochemical analysis of transverse spinal cord sections demonstrated diffuse expression of Hoxb4 throughout the spinal cord at E12.5 (C16/T19), after which expression was confined primarily to the presumptive gray matter.

4. At E14.25 (C19+/T21), $Olig2^+$ cells had begun to migrate out from the ventral ventricular zone into the presumptive gray matter. These results suggest that $Olig2^+$ cells could coexpress Hoxb4 since it is expressed throughout this region.

5. The expression of Hoxb4 by cells of the OG lineage indicates that it could play a role in OG maturation.

KEY WORDS: oligodendrocyte development; Hoxb4; Olig2; primary glial cultures; spinal cord.

INTRODUCTION

Oligodendrocytes (OGs) are myelinating cells of the central nervous system (CNS). In the murine spinal cord platelet-derived growth factor alpha receptor $(PDGF\alpha R)^+$ OG precursor cells (OPCs) first appear at approximately embryonic day (E) 12.5 in a distinct region of the ventral ventricular zone (vVZ) (Sun *et al.*, 1998). This region has been shown to correspond to the progenitor motor neuron domain (pMN), which is defined by the expression of the basic helix-loop-helix transcription factor Olig2 (Novitch *et al.*, 2001; Zhou *et al.*, 2000). Although Olig2 initially specifies motor neuron precursors at early stages of spinal cord development, it is downregulated as

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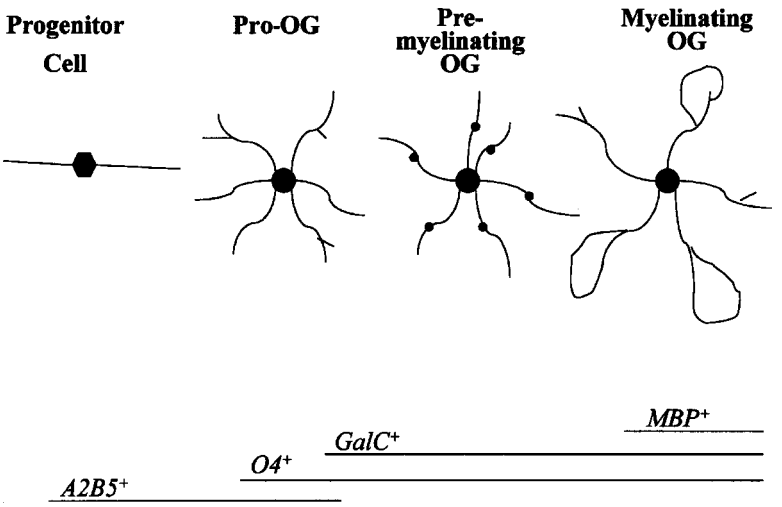

Fig. 1. Illustration depicting OG development. OGs progress through four distinct stages which are characterized by unique morphological and antigenic phenotypes (Abney *et al.*, 1983; Asou *et al.*, 1995; Bansal *et al.*, 1989; Duchala *et al.*, 1995; Fok-Seang and Miller, 1994; reviewed in Miller, 1996; Noble *et al.*, 1988; Raff *et al.*, 1983, 1985; Sommer and Schachner, 1981).

these precursor cells differentiate into motor neurons (Mizuguchi *et al.*, 2001; Novitch *et al.*, 2001; Zhou *et al.*, 2000). Olig2 expression that persists in the pMN domain after motor neurons are generated specifies cells of the OG lineage (Lu *et al.*, 2000; Zhou *et al.*, 2000). Initially only a few OPCs are found on each side of the central canal but these cells subsequently proliferate and migrate first laterally then dorsally to populate the entire cross-sectional area of the spinal cord (Lu *et al.*, 2000; Zhou *et al.*, 2000).

Analysis of glial cell differentiation in vitro has shown that OG development progresses through a number of distinct stages, which are characterized by the expression of specific cell surface markers (Fig. 1). During early stages of differentiation, characterized by A2B5 and/or O4 staining, cells are actively proliferating and migratory. Loss of these traits signals terminal differentiation of the OG lineage, which is characterized by the expression of galactocerebroside (GalC) and subsequently the expression of myelin proteins (Abney *et al.*, 1983; Asou *et al.*, 1995; Bansal *et al.*, 1989; Duchala *et al.*, 1995; Fok-Seang and Miller, 1994; reviewed in Miller, 1996; Noble *et al.*, 1988; Raff *et al.*, 1983, 1985; Sommer and Schachner, 1981).

The timing of OG differentiation is believed to be dependent on an intrinsic clock that counts cell divisions driven by mitogens, such as platelet-derived growth factor (PDGF) (Raff *et al.*, 1985, 1988). Differentiation is further modified by hydrophobic signals, such as retinoic acid (RA) or thyroid hormone (Barres *et al.*, 1994; Ibarrola *et al.*, 1996; Noll and Miller, 1994). Potential downstream targets of RA signaling are the *Hox* genes, which are characterized by a 180-bp homeobox (Gould *et al.*, 1998; McGinnis *et al.*, 1984a,b). This nucleotide sequence encodes a 60 amino acid homeodomain that contains a helix-turn-helix motif (Kissinger *et al.*, 1990; McGinnis *et al.*, 1984a,b). Hence *Hox* genes can regulate the expression of downstream effector

genes by binding to specific nucleotide sequences through the homeodomain (Han *et al.*, 1989; Hoey and Levine, 1988; Kissinger *et al.*, 1990). *Hoxb4* is one of 39 mouse and human *Hox* genes that are organized into four clusters (Hox A, B, C, D) located on different chromosomes (Favier and Dollé, 1997; Graham *et al.*, 1989; Scott, 1992). In situ hybridization histochemistry conducted by Gaunt *et al.* (1989) and Graham *et al.* (1988, 1989) demonstrated that *Hoxb4* mRNA is expressed in numerous tissues, including the CNS with the anterior limit of expression extending into the myelencephalon. We have analysed Hoxb4 expression patterns during murine OG development in cell cultures and in spinal cord sections. Our results demonstrate that Hoxb4 is expressed by cells of the OG lineage.

MATERIALS AND METHODS

Primary Glial Cultures

Cerebral hemispheres from newborn mice were dissected in DMEM/F12/10% FBS. Single cell suspensions, obtained by forcing diced tissue through a 75 μm Nitex mesh, were plated in DMEM/F12/10% FBS for 4 days to allow adherence of the cells. They were then cultured for two 1-week periods, respectively in a B104 conditioned medium and a low serum-containing (0.3% FBS) growth medium (GP medium) used previously by Gard and Pfeiffer (1990) and by Doucette and Devon (1994); this OG growth medium was supplemented with 10 ng/mL basic fibroblast growth factor (bFGF). The growth medium was changed every 4 days.

Immunocytochemistry

Immunocytochemistry utilized the following primary antibodies: anti-Hoxb4 (BabCO, CA; 1:500), A2B5 (ATCC; 1:10), O4 (hybridoma; Sommer and Schachner, 1981; 1:10), anti-galactocerebroside (GalC) (BRD1 hybridoma; Ranscht *et al.*, 1982; 1:20), and anti-myelin basic protein (MBP) (Sternberger Monoclonals; 1:800). The cell cultures were double immunostained with Hoxb4 and A2B5, O4, or GalC using the live staining method described by Doucette and Devon (1994) and Hao *et al.* (1999) but with an incubation period of 30 min at 37°C for A2B5, O4, and anti-GalC. The secondary antibodies used included a goat anti-mouse IgM FITC (Sigma, ON; 1:100); a donkey anti-mouse IgG FITC (BIO/CAN Scientific, ON; 1:50); and a goat anti-rabbit IgG CY3 (BIO/CAN Scientific, ON; 1:200). Between incubations cultures were washed two times for 5 min each in PBS. Double immunostaining for Hoxb4 and MBP was done using the procedure described in Doucette and Devon (1994) with two modifications: (1) permeabilization and blocking were combined with a 30 min incubation in 3% skim milk/0.1% Triton-X; (2) the primary antibodies were diluted in 1% skim milk/0.03% Triton-X. The nuclei in all cell cultures were stained using HOECHST dye.

Immunohistochemistry

C57 black mouse embryos were staged according to the Carnegie (Butler and Juurlink, 1987) and Theiler (1972) staging systems. The embryonic ages (E) and

corresponding Carnegie(C)/Theiler (T) stages utilized were E12.5 [C16/T19], E14.25 [C19+/T21], E16 [C23/T23], and E18 [T25]; the Carnegie staging system does not go higher than stage 23. Once the embryos were staged, they were fixed by immersion in 4% paraformaldehyde at 4°C for 24–48 h depending on their age, and cryostat sections (8 μm) were cut through the upper thoracic level as described by Hao *et al.* (1999). The following primary antibodies were used: polyclonal anti-Olig2 (E12.5, 1:4000; rest of stages, 1:2000, gift of Dr Hirohide Takebayashi) and anti-Hoxb4 (E12.5, 1:3000; rest of the stages, 1:2000). Immunohistochemical staining was performed using the procedure described by Hao *et al.* (1999), with the following modifications: PBS washes were 8 min instead of 10 min; 3% skim milk/0.1%Triton-X block/permeabilizations were 20 min versus 30–60 min; and incubation in the secondary antibody was 30 min versus 45 min.

RESULTS AND DISCUSSION

Immunocytochemistry was conducted in this study as it allowed for a thorough investigation of Hoxb4 expression throughout OG development. The cerebral hemisphere cultures analyzed consisted of oligodendroglia that developed on top of an astrocytic monolayer. Although the anterior limit of *Hoxb4* expression had been shown to be the mid-myelencephalon (Gaunt *et al.*, 1989; Graham *et al.*, 1988, 1989), Hoxb4 was expressed by cells obtained from murine cerebral hemispheres. Similar findings have been observed with Hoxa2 where its expression was subsequently shown to extend to the forebrain (Hao *et al.*, 1999; Tan *et al.*, 1992; Wolf *et al.*, 2001). Furthermore our laboratory had previously demonstrated that Hoxa2 was expressed by $O4^+$ pro-OGs obtained from E15 mouse cerebral hemispheres (Hao *et al.*, 1999).

Hoxb4 was expressed by the majority of cells throughout OG development. Greater than 90% of the cells that expressed O4 (97%), GalC (91%), and MBP (93%) also coexpressed Hoxb4 (Fig. 2). The percentage of $A2B5^+$/$Hoxb4^+$ cells was not determined because A2B5 labels oligodendrocyte type 2 astrocyte (O-2A) precursor cells, which can give rise to either OGs or type 2 astrocytes depending on culture conditions (Raff *et al.*, 1983), and therefore is not specific to the OG lineage. Expression of Hoxb4, which is a transcription factor, was primarily nuclear (Fig. 2(B, E, K)), however weak cytoplasmic staining was observed occasionally in $GalC^+$ cells (Fig. 2(H)). The reason for this is unknown but similar observations have been reported with several other transcription factors (Armstrong *et al.*, 1995; Wang *et al.*, 2001).

Although Hoxb4 was expressed throughout OG development, it was not specific to the OG lineage. This was demonstrated by the fact that the number of $Hoxb4^+$ cells was greater than that of OG markers in primary mixed glial cultures (Fig. 2). Furthermore *Hoxb4* RNA has been shown to be expressed in various embryonic tissues, including hindbrain, spinal cord, stomach, lung, kidneys, and prevertebra (Gaunt *et al.*, 1989; Graham *et al.*, 1988).

Hoxb4 expression was investigated in transverse spinal cord sections at stages E12.5 [C16/T19] to E18 [T25]. At E12.5 [C16/T19] expression was diffuse being slightly more intense in the ventral horns (Fig. 3(A)). At later stages of development

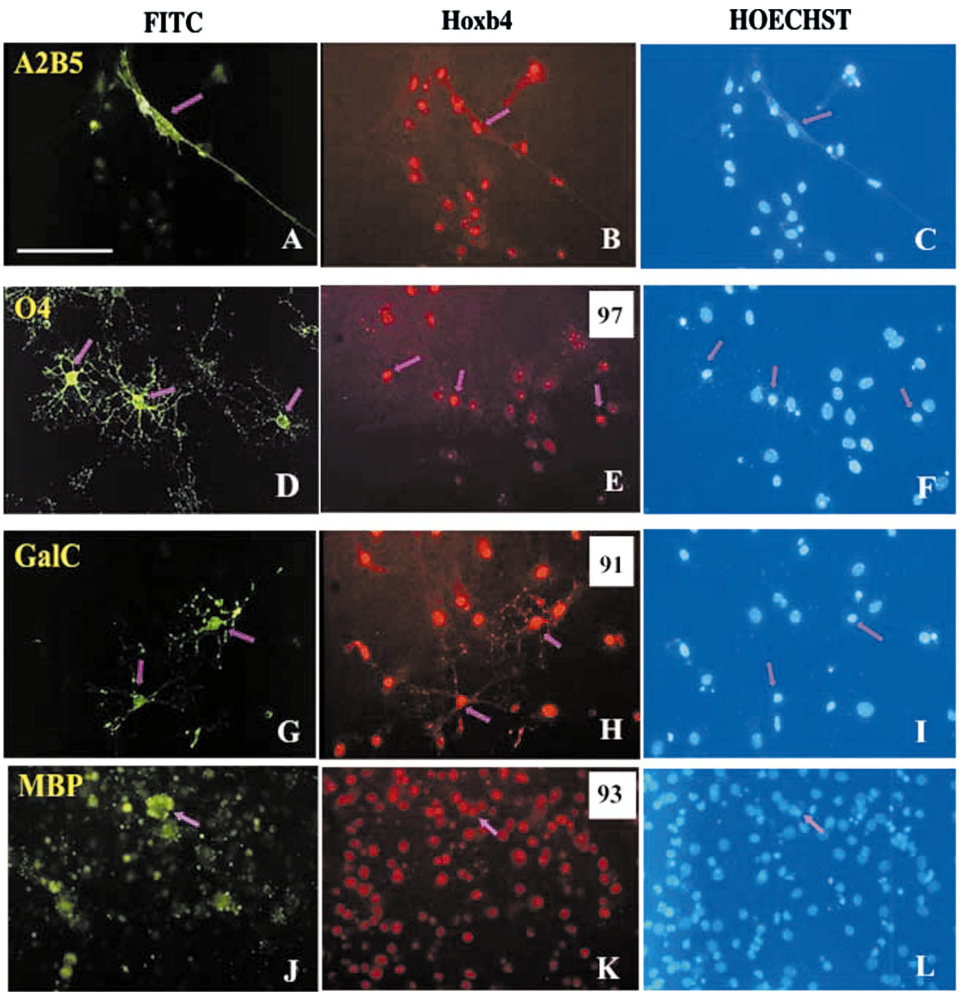

Fig. 2. Hoxb4 expression pattern throughout OG development. Primary glial cell cultures from neonatal murine brain were grown in culture for 18 days. Double immunofluorescent staining was conducted with each of the four OG markers [A2B5 (A), O4 (D), GalC (G), and MBP (J)] and Hoxb4 (B, E, H, K). Cells were visualized utilizing nuclear fluorescent HOESCHT counterstain. Each row illustrates micrographs obtained from individual filters [fluorescein (A, D, G, J), rhodamine (B, E, H, K), and DAPI (C, F, I, L)] of the same field. Arrows demarcate representative Hoxb4 immunoreactive cells. The insets at the top right corner of the rhodamine micrographs (E, H, L) show the percentage of $O4^+$, $GalC^+$, and MBP^+ cells that are immunoreactive for Hoxb4, respectively. [Percentages depict averages calculated from 2 to 3 coverslips in which 100–150 OGs were counted.] Bar = 100 μm for Fig. (A)–(L).

(E14.25–E18) Hoxb4 was expressed primarily in the presumptive gray matter (Fig. 3(C, E, G)). Previously, Graham *et al.* (1988, 1991) and Gaunt *et al.* (1989), had determined by in situ hybridization histochemistry the expression patterns of *Hoxb4*. Although only one micrograph of a ~12.5 day postcoital (d.p.c) transverse spinal cord section illustrated *Hoxb4* expression, it had been stated to exhibit a dorsoventral expression pattern similar to that of the other *Hoxb* genes (Graham *et al.*, 1988, 1991). At

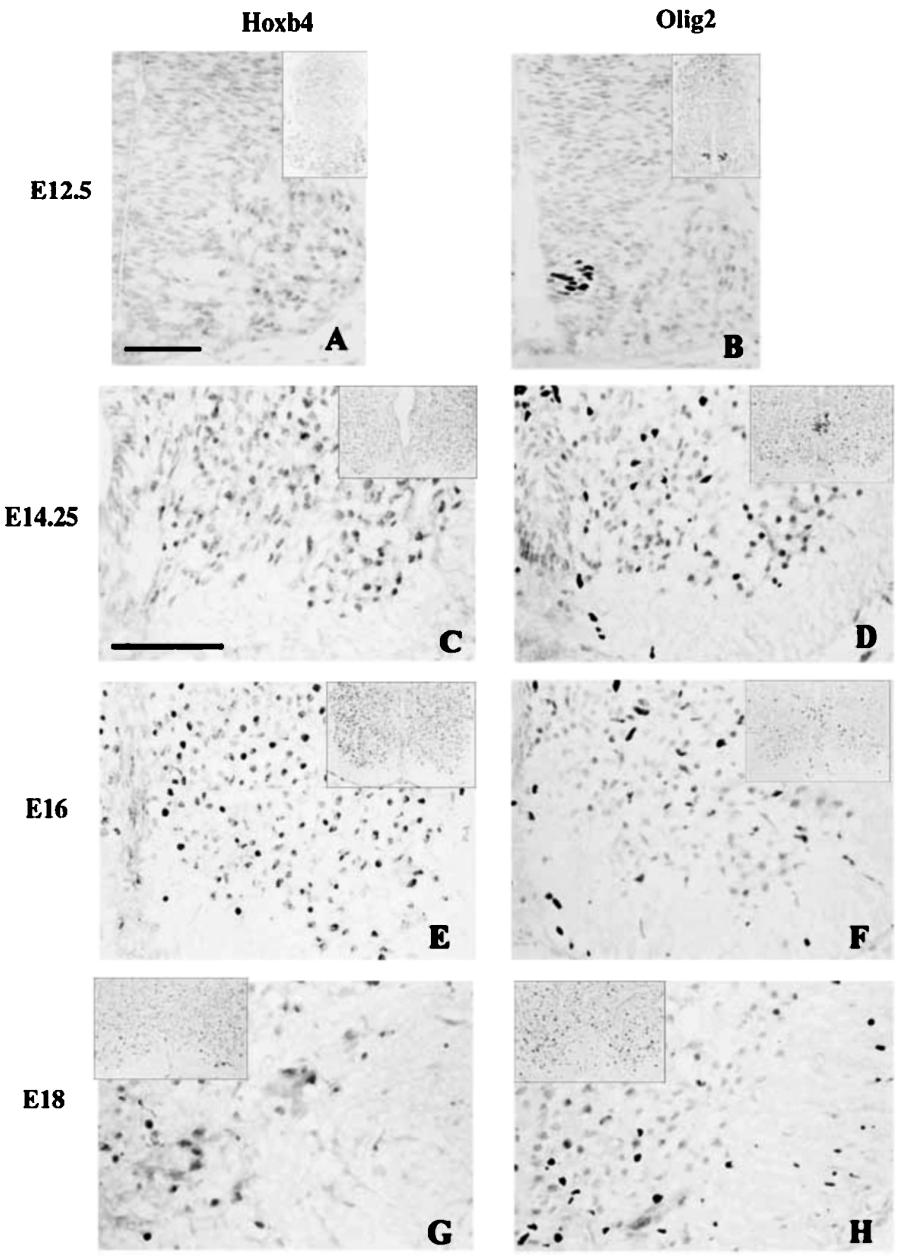

Fig. 3. Expression of Hoxb4 in the embryonic spinal cord in relation to the OG marker Olig2. Transverse sections of the thoracic spinal cord at E12.5 [C16/T19], E14.25 [C19+/T21], E16 [C23/T23], and E18 [T25] were immunolabeled with Hoxb4 (A, C, E, G) and Olig2 (B, D, F, H). At E12.5 when Olig2$^+$ OPCs (B) were localized to the vVZ Hoxb4 (A) was diffusely expressed throughout the spinal cord, being slightly more intense in the ventral horns. Also at later stages of embryonic development Hoxb4's expression pattern (C, E, G) displayed domains of overlap with Olig2 (D, F, H). The micrographs are representative of sections obtained from approximately four embryos from at least two different litters. Bar = 100 μm for Fig. (A)–(H).

10.5 d.p.c., the expression of *Hoxb* genes was dispersed throughout the entire spinal cord (Graham *et al.*, 1988, 1991). In our immunohistochemical analysis, immunoreactivity appeared to be slightly greater in the ventral horns (Fig. 3(A)). In situ hybridization histochemistry has also shown that *Hoxb* genes exhibit strong expression in the dorsal spinal cord at 12.5 d.p.c. after which expression appeared in the ventral spinal cord at 14.5 d.p.c. (Graham *et al.*, 1991). In our study, Hoxb4 was found to be expressed throughout the presumptive gray matter at E14.25 [C19+/T21] (Fig. 3(C)). The *Hoxb4* gene has been shown to give rise to multiple transcripts (Graham *et al.*, 1988); it is not known if all yield a translated product. The probe utilized by Graham *et al.* (1991) did not distinguish between them; therefore, differences in expression patterns may exist for the individual transcripts.

There are currently very few immunohistochemical markers available for studying OG development in vivo. Therefore, OG development in the spinal cord was followed by analyzing Hoxb4 expression in relation to that of Olig2. Evidence that supports the use of Olig2 as an OG marker is threefold. First, Olig2$^+$ cells, which are initially found in pMN domain, disperse throughout the spinal cord as previously described for PDGFαR (Lu *et al.*, 2000; Zhou *et al.*, 2000). Furthermore, it has been shown that the number of Olig2$^+$ cells is similar to that of PDGFαR$^+$ cells (Zhou *et al.*, 2000). Second, Olig2 is not coexpressed with the astrocytic marker glial fibrillary acidic protein (GFAP) (Takebayashi *et al.*, 2000). Finally, Olig2 appears to be critical for OG development since in its absence OPCs and differentiated OGs fail to develop in the spinal cord (Lu *et al.*, 2002). Therefore, Olig2 is the earliest known marker of OG development since its expression precedes that of PDGFαR by approximately 1–1.5 days (Zhou *et al.*, 2000). Immunohistochemical analysis in this report has focused primarily on the ventral spinal cord due to the predominant expression of Olig2 in this region during embryonic development. We were unable to demonstrate coexpression of Hoxb4 with Olig2 since both antibodies were rabbit polyclonals.

Immunohistochemical analyses were initiated at E12.5 [C16/T19] since at this stage Olig2 expression has been downregulated in differentiated motor neurons and PDGFαR$^+$ OPCs have arisen in the vVZ. Unlike Olig2, which was localized to the vVZ (Fig. 3(B)), Hoxb4 stained nuclei were diffusely distributed at this stage, with immunoreactivity slightly more intense in ventral horns (Fig. 3(A)). The Hoxb4$^+$ cells in the ventral horns were most likely neurons because neuron-specific β-tubulin was also expressed in these areas (data not shown). However, the results suggest that Hoxb4 may be expressed by OPCs in vivo since Hoxb4 stained nuclei are diffusely distributed throughout the ventricular zone. From E14.25 [C19+/T21] onward Hoxb4 was expressed throughout the presumptive gray matter (Fig. 3(C, E, G)). Beginning at E16 [C23/T23], some Hoxb4$^+$ cells were also observed in the presumptive white matter, increasing somewhat in numbers at E18 [T25] (Fig. 3(E, G)). Olig2$^+$ cells began to accumulate in the presumptive gray matter at ~E14.25 [C19+/T21] with occasional immunoreactive cells found in the presumptive white matter (Fig. 3(D)). This migratory pattern continued at later stages with an increasing number of immunoreactive cells accumulating in the presumptive white matter (Fig. 3(F, H)). Therefore, these results suggest that a population of Olig2$^+$ cells may coexpress Hoxb4. However, the Olig2$^+$ cells outnumbered Hoxb4$^+$ in the presumptive white

matter (Fig. 3(G, H)). In addition, the number of Hoxb4$^+$ cells in transverse spinal cord sections was greater than that of the Olig2$^+$ cells, which lends support to our cell culture findings of cells other than OG expressing this Hox protein.

One potential role of Hoxb4 in OG development may be as a downstream effector in RA pathway. The role of RA in OG development has previously been described. In particular, Noll and Miller (1994), utilizing E14–18 rat spinal cord cultures, found that RA (1 μM) inhibits maturation of OPCs. Interestingly, Barres *et al.* (1994) showed that low concentrations of RA (10 nm) applied to postnatal day 8 rat optic nerve cultures promoted differentiation. Although these results initially appear to be contradictory it could be hypothesized that they represent ends of the same developmental pattern. Hence high concentrations of RA early on in development could inhibit OG differentiation, allowing OPC distribution throughout the spinal cord (Noll and Miller, 1994). Then as OG development proceeds and RA levels diminish, cells could be facilitated to differentiate. Since RA has been shown to regulate Hoxb4 expression in hindbrain (Gould *et al.*, 1998), it has the potential to affect expression in OGs. Now that it has been demonstrated that Hoxb4 is expressed by cells of the OG lineage it will be interesting to determine whether RA exerts its effects on OG development via *Hox* genes, such as *Hoxb4*.

ACKNOWLEDGMENTS

Supported by a research grant from the Canadian Institutes of Health Research to A.J.N and J.R.D. We are grateful to the Multiple Sclerosis Society of Canada for support for technical personnel and to Ms. Sheng Yu for technical assistance with primary cell culture preparations. DJN is supported by an Rx&D/CIHR scholarship. We extend our gratitude to Dr. Hirohide Takebayashi for the Olig2 antibody.

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Transcriptional Control of Oligodendrogenesis

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KEY WORDS
oligodendrogenesis; transcription factors; specification; maturation; signaling pathways

ABSTRACT
Oligodendrocytes (OGs) assemble the myelin sheath around axons in the central nervous system. Specification of cells into the OG lineage is largely the result of interplay between bone morphogenetic protein, sonic hedgehog and Notch signaling pathways, which regulate expression of transcription factors (TFs) dictating spatial and temporal aspects of oligodendrogenesis. Many of these TFs and others then direct OG development through to a mature myelinating OG. Here we describe signaling pathways and TFs that are inductive, inhibitory, and/or permissive to OG specification and maturation. We develop a basic transcriptional network and identify similarities and differences between regulation of oligodendrogenesis in the spinal cord and brain.

INTRODUCTION

In the central nervous system (CNS), oligodendrocytes (OGs) assemble the myelin sheath around axons (Southwood et al., 2004). OG development *in vitro* progresses through distinct stages characterized by specific antigenic phenotypes (Fig. 1). During early stages of oligodendrogenesis, A2B5$^+$ oligodendrocyte precursor cells (OPCs) and O4$^+$ pro-OGs actively proliferate (Fok-Seang and Miller, 1994) and migrate (Noble et al., 1998). Subsequently, loss of these traits and the emergence of galactocerebroside (GalC) expression (Fok-Seang and Miller, 1994; Noble et al., 1998) signal terminal differentiation into premyelinating OGs. Progression to the mature myelinating phenotype occurs when cells synthesize myelin proteins and elaborate sheet-like membranes (Duchala et al., 1995).

In developing vertebrate embryos, OPCs arise in multiple distinct foci located in various regions of the CNS (Cai et al., 2005; Kessaris et al., 2006; Perez Villegas et al., 1999; Pringle and Richardson, 1993; Spassky et al., 1998). Subsequently, oligodendroglial cells from these foci migrate out to populate specific regions of the CNS (Kessaris et al., 2006; Ono et al., 1997; Pringle and Richardson, 1993; Spassky et al., 1998; Tekki-Kessaris et al., 2001). Interestingly, OG development and ultimately myelination progress at different rates dependent upon the CNS region examined (Fok-Seang and Miller, 1994; Foran and Peterson, 1992; Hamano et al., 1998; LeVine and Goldman, 1988; Reynolds and Wilkin, 1988).

A very dynamic combination of transcription factors (TFs) are expressed by OGs as they progress from precursor to mature myelinating cells (Fig. 1). Whereas some TFs, such as Sox10 (Stolt et al., 2002), are expressed throughout OG development, expression of many TFs, such as SCIP (Collarini et al., 1992) and Krox24 (Sock et al., 1997), is transient. In most cases expression decreases with differentiation (Collarini et al., 1992; Schreiber et al., 1997); however, for Nkx6.2, expression increases in time with that of myelin proteins (Awatramani et al., 1997). Role(s) of some TFs have been elucidated utilizing transgenic knockout mice and transfection assays (Table 1). In some cases TF function is consistent with its expression profile as is the case with Olig2, which is expressed throughout OG development (Lu et al., 2000) and is important in OG specification (Lu et al., 2002) and maturation (Fu et al., 2002). However, in other cases the expression profile may not easily suggest the TF function. For example, Sox10 is expressed throughout OG development but is only critical in OG maturation (Stolt et al., 2002). Interestingly, most of these TFs are downstream targets or effectors in signaling pathways, such as sonic hedgehog (Shh) (Ericson et al., 1997; Lu et al., 2000), bone morphogenetic protein (BMP) (Samanta and Kessler, 2004), and Notch (Wang et al., 1998).

This review describes transcriptional control of oligodendrogenesis by examining TFs not in isolation but in the context of a transcriptional network. Although one can make fairly confident statements about roles of several TFs in oligodendrogenesis, functional linkages between TFs and/or signaling pathways require more conjecture. Our approach to this complex problem is to discuss TFs and signaling pathways in the context of their roles as inductive, inhibitory and/or permissive

Grant sponsors: Canadian Institutes of Health Research (CIHR), Multiple Sclerosis Society of Canada.

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Received 16 January 2007; Revised 29 May 2007; Accepted 4 June 2007

DOI 10.1002/glia.20540

Published online 00 Month 2007 in Wiley InterScience (www.interscience.wiley.com).

J_ID: Z76 A_ID: 00015-2007.R2 Art: GLIA20540 Date: 21-JUNE-07 Stage: I Page: 2

agents to OG specification and maturation. In doing so, the review points out known, unknown and unresolved issues in the transcriptional control of oligodendrogenesis and hypothesizes potential roles or connections of the TFs and signaling pathways in question. The synthesis of information regarding these TFs and signaling pathways into a transcriptional network serves as a template to be modified as new information is obtained and as a resource to researchers in formulating pertinent hypotheses and designing appropriate experimental protocols.

OG SPECIFICATION
Inductive Factors

In the embryonic spinal cord, platelet-derived growth factor α-receptor (PDGFαR)$^+$ OPCs initially arise from a distinct region of the ventral ventricular zone (vVZ) called the motor neuron progenitor (pMN) domain (Fu et al., 2002) (Fig. 2). The pMN domain is specifically demarcated by the basic helix-loop-helix (bHLH) TF Olig2 (Fu et al., 2002; Novitch et al., 2001). Olig2 is critical to OG specification in the spinal cord since PDGFαR expression is lost in its absence (Lu et al., 2002). Furthermore, Nkx6.1 (Liu et al., 2003) and Gli2 (Qi et al., 2003) mutant mice, which display a dramatic transient decrease in Olig2 expression, correspondingly exhibit a delay and transient reduction in PDGFαR expression (Liu et al., 2003; Qi et al., 2003). Olig1 is also expressed in the pMN domain of rodents (Zhou et al., 2000) but is not critical to OG specification in the spinal cord since PDGFαR expression appears on schedule in its absence (Lu et al., 2002).

Expression of Olig1 and Olig2 is induced by Shh in a concentration-dependent manner (Lu et al., 2000). In the early embryonic spinal cord Shh exhibits a ventral to dorsal decreasing expression gradient (Gritli-Linde et al., 2001) (Fig. 2) that directs formation of five ventral progenitor domains, including the pMN domain, by regulating expression of TFs in the vVZ (Briscoe et al., 2000; Ericson et al., 1997; Lu et al., 2000). Hence Shh is important to OG specification in the spinal cord since it regulates Olig2 expression. Indeed, Wnt-1/Shh transgenic mice, which ectopically express Shh in the dorsal midline of the spinal cord under the control of the Wnt-1

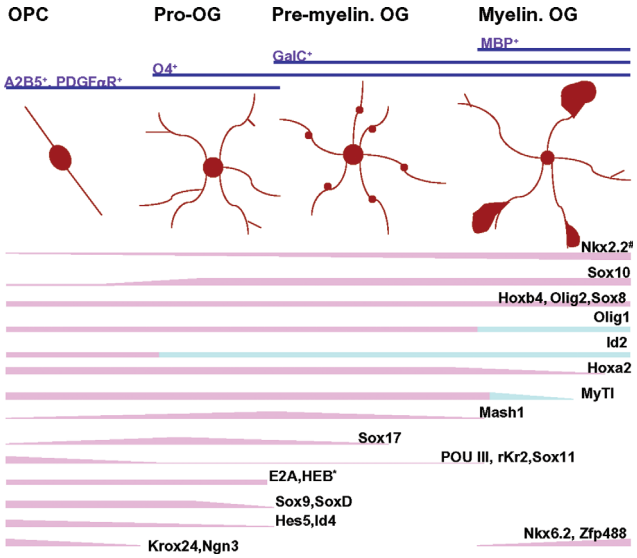

Fig. 1. Expression profiles of various TFs during OG development. (*top*) OGs progress through four distinct stages characterized by unique morphological and antigenic phenotypes. (myelin.: myelinating). (*bottom*) Speculated expression profiles of TFs expressed by cells of the oligodendroglial lineage (see Table 1 for references). If not stated otherwise, expression was assumed to be nuclear. In cases where overall expression profiles of two or more TFs appeared similar, TFs were grouped together (e.g., Hes5 and Id4). Variations in line thickness illustrate either changes in the percentage of cells expressing the TF(s) or the intensity of the TF(s) expression. (—), nuclear expression; (—), cytoplasmic expression; POU III, Brn-1,2 and SCIP; *, later stages of OG development were not analyzed; #, expression profile may vary depending upon the CNS region and/or cell line.

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Abbreviations

bHLH	Basic helix-loop-helix
BMP	Bone morphogenetic protein
Brn	Brain
CBF1	C promoter binding factor 1
CNP	2′,3′-cyclic nucleotide 3′-phosphodiesterase
CNS	Central nervous system
COUP-TFI	Chicken ovalbumin upstream promoter-transcription factor I
Dpc	Days postcoitum
E	Embryonic age
E2F-1	E2F transcription factor 1
FGF	Fibroblast growth factor
GalC	Galactocerebroside
GABA	Gamma-aminobutyric acid
GFP	Green fluorescent protein
Gli2	GLI-Krűppel family member Gli2
Hes5	Mammalian homolog of *hairy* and *Enhancer of split*
Hox	Homeobox
Id	Inhibitor of DNA binding
Irx	Iroquois
Krox24	Krüppel box
MAG	Myelin associated glycoprotein
Mash1	Mammalian *achaete-scute* homolog 1
MBP	Myelin basic protein
MN	Motor neuron
MyTI	Myelin transcription factor I
NFIA	Nuclear factor I A
Ngn	Neurogenin
Nkx2.1/Nkx2.2	NK2 transcription factor related, locus 1 or 2
Nkx6.1/Nkx6.2	NK6 transcription factor related, locus 1 or 2
OG(s)	Oligodendrocyte(s)
Olig	Oligodendrocyte transcription factor
OPC(s)	Oligodendrocyte precursor cell(s)
P	Postnatal day
Pax6	Paired box gene 6
PDGFαR	Platelet-derived growth factor alpha receptor
PLP	Proteolipid protein
pMN	Motor neuron progenitor domain
POU	Pit-1, OTF1 & 2, UNC86 family of genes
PSA-NCAM	Polysialylated neural cell adhesion molecule
RA	Retinoic acid
RAR	Retinoic acid receptor
RXR	Retinoid X receptor
SCIP	Suppressed cAMP inducible POU
Shh	Sonic hedgehog
siRNA	Short interfering RNA
Sox	SRY-box containing gene
TF(s)	Transcription factor(s)
TH	Thyroid hormone
T_3	triiodothyronine
T_4	Thyroxine
vVZ	Ventral ventricular zone
Wnt-1	Wingless-type MMTV integration site family, member 1
Zfp488	Zinc finger protein for OG differentiation.

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TABLE 1. TFs Expressed by Cells of the OG Lineage

Family	Class/group	Transciption factor	Role in OG specification	Role in OG maturation	Additional CNS expressing cells
HLH	I	E2A[36]			AQ3
		HEB[36]			
	II	Mash1[12,39]			Neural precursor[18]
		Ngn3[17]	Sp (SC)[17]	+[17]	Neural precursor[17]
		Olig1[4,21]	+ (B)[20,21,41]	+[20,40]	Neural precursor[21]
		Olig2[4,8]	+[20#]	+[8]	Neural precursor[21,26]
	V	Id2[39]		−[13,39]	Neural precursor, neuron[11,22], astrocyte[37]
		Id4[13]			Neural precursor, neuron[11]
	VI	Hes5[12]		−[12]	Neural precursor, neuron[1]
Hox	A	Hoxa2[9,23]			Neuron[9,23], astrocyte[9]
	B	Hoxb4[24]			Neuron[24]
Nkx		Nkx2.2*[27]	Sp (SC)[27]	+[27]	Neural precursor[27]
		Nkx6.2*[5]		Myelin (u)[31]	Neural precursor, neuron[31]
POU	III	Brn-1[28]			Neural precursor[2], neuron[10]
		Brn-2[28]			Neural precursor[2], neuron[10]
		SCIP[7,28]			Neural precursor, neuron[2]
Sox	C	Sox4[15]			Neural precursor, neuron[6]
		Sox11[15]			Neural precursor, neuron[6,15]
	D	Sox5[32]	Tp (SC)[32]	−[32]	Neural precursor, neuron, radial glia[32]
		Sox6[32]	Tp (SC)[32]	−[32]	Neural precursor, neuron, radial glia[32]
	E	Sox8*[33]		+[33]	
		Sox9[34a]	+s (SC)[34]		Neural precursor, radial glia, astrocyte[34], bergmann glia[14]
		Sox10[16]*[35]		+[35]	
	F	Sox17[30]		+[30]	Neuron, astrocyte[30]
Zinc finger		Krox24[29]			
		MyTI[3]*[25]		+[25]	Neural precursor, neuron[3]
		Zfp488[38]		+[38]	
	Krüppel	rKr2[19]			Neural precursor, neuron[19]

HLH, helix-loop-helix; +, promote; −, inhibit; Sp, spatial; B, brain specific effect; SC, spinal cord; #, specific brain regions not affected; *, TF binding sites found in the promoters of myelin protein genes; Tp, temporal; (u), ultrastructure; +s, promotes neural to glial switch.
References: [1]Akazawa et al. (1992), [2]Alvarez-Bolado et al. (1995), [3]Armstrong et al. (1995), [4]Arnett et al. (2004), [5]Awatramani et al. (1997), [6]Cheung et al. (2000), [7]Collarini et al. (1992), [8]Fu et al. (2002), [9]Hao et al. (1999), [10]He et al. (1989), [11]Jen et al. (1996), [12]Kondo and Raff (2000a), [13]Kondo and Raff (2000b), [14]Kordes et al. (2005), [15]Kuhlbrodt et al. (1998a), [16]Kuhlbrodt et al. (1998b), [17]Lee et al. (2003), [18]Lo et al. (1991), [19]Lovas et al. (2001), [20]Lu et al. (2002), [21]Lu et al. (2000), [22]Neuman et al. (1993), [23]Nicolay et al. (2004a), [24]Nicolay et al. (2004b), [25]Nielsen et al. (2004), [26]Novitch et al. (2001), [27]Qi et al. (2001), [28]Schreiber et al. (1997), [29]Sock et al. (1997), [30]Sohn et al. (2006), [31]Southwood et al. (2004), [32]Stolt et al. (2006), [33]Stolt et al. (2004), [34]Stolt et al. (2003), [35]Stolt et al. (2002), [36]Sussman et al. (2002), [37]Tzeng and De Vellis (1998), [38]Wang et al. (2006), [39]Wang et al. (2001), [40]Xin et al. (2005), [41]Zhou and Anderson (2002).

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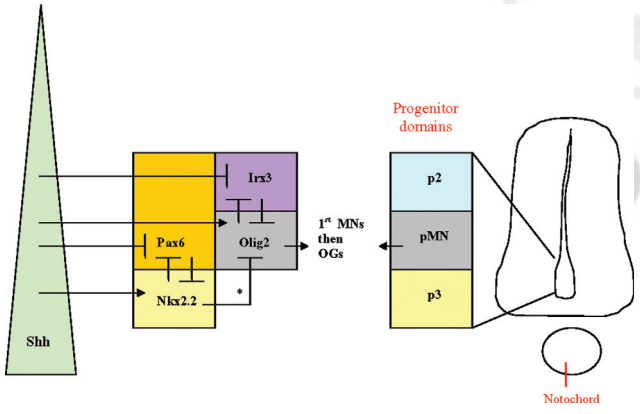

Fig. 2. Shh signaling directs the formation of ventral progenitor (p) domains, such as the pMN domain, which gives rise to motor neurons (MNs) and subsequently oligodendroctyes (OGs). Shh, which exhibits a ventral to dorsal decreasing expression gradient in the spinal cord, enhances (→) or represses (⊥) the expression of the TFs defining the boundaries of the pMN domain. These boundaries are further maintained and refined by repressive interactions between these TFs. (*, effect is only evident during early stages of development). NB: Whether MNs and OGs arise from a common precursor or separate neural- and glial-restricted precursors remains unresolved.

regulatory element, exhibit ectopic Olig2 and PDGFαR expression in this region (Lu et al., 2000).

Recent research shows the role of Shh is consistent throughout most of the CNS. In particular, injection of a Shh-expressing retrovirus into embryonic day (E) 9.5 murine telencephalic vesicles results in ectopic Olig2 and PDGFαR expression (Nery et al., 2001). Conversely, Shh mutant mice lack Olig1, Olig2 (Alberta et al., 2001; Lu et al., 2000), and PDGFαR (Alberta et al., 2001) expression throughout the brain. Furthermore, in Nkx2.1 mutant mice, which lack Shh expression in the medial ganglionic eminence and the hypothalamus at E11.5 (Sussel et al., 1999), telencephalic expression of PDGFαR is absent at E14.5 (Nery et al., 2001; Tekki-Kessaris et al., 2001).

Although Shh is crucial to OG specification throughout much of the CNS by regulating expression of Olig TFs, the function(s) of these TFs vary by region. Unlike the spinal cord, PDGFαR expression is evident in the hindbrain and in focal regions of the ventral forebrain in the absence of Olig2 (Lu et al., 2002). However, in Olig1/2 double knockout mice, PDGFαR expression is absent in all examined brain regions (Zhou and Anderson, 2002). Therefore, Olig1 is important in OG specification in certain brain regions. Accordingly, infection of cortical cultures with an Olig1-containing adenovirus greatly increases the percentage of infected cells that are OPCs (Alberta et al., 2001; Lu et al., 2000). As researchers have yet to identify the chick ortholog of Olig1 (Zhou et al., 2001), the TF Nkx2.2, whose expression is also induced by Shh in a concentration-dependent manner (Ericson et al., 1997), may play an important role in OG specification in certain chick brain regions. A supportive finding is that in the embryonic chick hindbrain only Nkx2.2 is expressed in the vVZ during OG

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specification whereas Olig2 is expressed by groups of Nkx2.2$^+$ cells in the subventricular zone and in individual migrating cells (Fu et al., 2002). Also, recent research suggests that Mash1 is important for the specification of a particular population of OPCs in the ventral forebrain since in its absence a dramatic transient decrease in PDGFαR expression occurs in this area at E12.5 and E13.5 (Parras et al., 2007).

Spatial and Temporal Modulating Factors

Although Olig1 and Olig2 specifically demarcate the pMN domain (Fu et al., 2002; Zhou et al., 2000), three additional TFs—Nkx2.2, Pax6, and Irx3—define its boundaries (Briscoe et al., 2000) (Fig. 2). In particular, the ventral boundary of the pMN domain is defined by Pax6 and Nkx2.2 (Briscoe et al., 2000), whereas the dorsal boundary is defined by Irx3 and Olig2 (Fu et al., 2002). Subsequently cross-repressive interactions between these TFs maintain and refine these boundaries (Briscoe et al., 2000; Novitch et al., 2001). In addition, at early stages of spinal cord development, Nkx2.2 also represses Olig2 expression (Novitch et al., 2001; Zhou et al., 2001).

Therefore, Nkx2.2, Pax6, and Irx3 should be important in spatial aspects of OG specification since they determine the boundaries of the Olig2$^+$ pMN domain. Furthermore, as early development appears to proceed from ventral to dorsal in the spinal cord (Altman and Bayer, 1984), these TFs could also be important in temporal aspects of OG specification. Indeed, Pax6-deficient mice exhibit a dorsal-ward shift and a delay of 1 day in the appearance of PDGFαR$^+$ cells (Sun et al., 1998). These anomalies likely occur since Pax6-deficient mice display a dorsal expansion in the expression domain of Nkx2.2 (Ericson et al., 1997) that represses Olig2 expression at early stages of development (Novitch et al., 2001; Zhou et al., 2001). Correspondingly, in the absence of Nkx2.2 a ventral expansion occurs in the Olig2$^+$ pMN domain (Qi et al., 2001). This ventral expansion also occurs in Ngn3 mutant mice, which lack Nkx2.2 expression in the spinal cord at E13.5 (Lee et al., 2003). Whether ventral expansion in the Olig2$^+$ domain results in premature OG specification in these mutant mice has yet to be determined. Interestingly, in the absence of both Nkx6.1 and Nkx6.2 TFs, a dorsal expansion of the Nkx2.2$^+$ domain occurs (Vallstedt et al., 2005), which may be responsible for the lack of PDGFαR expression in the pMN domain (Cai et al., 2005; Vallstedt et al., 2005) in these mutant mice. Although the reason for the dorsal expansion of the Nkx2.2$^+$ domain is unknown, it is likely indirect since Nkx2.2 and Nkx6.1/6.2 TFs exhibit overlapping expression domains in the vVZ during early stages of spinal cord development (Briscoe et al., 2000; Vallstedt et al., 2001).

The function of Irx3 in OG development has not been investigated but is likely important in temporal and spatial aspects of OG specification since Irx3 represses Olig2 expression (Novitch et al., 2001). In particular, the absence of Irx3 could result in a dorsal expansion in the Olig2$^+$PDGFαR$^+$ domain as well as prolonged OG specification. Whether Irx3, as well as Nkx2.2 and Pax6, could play similar roles in the brain, or whether additional TFs are involved has not been determined.

Inhibitory Factors

Unlike Shh signaling, BMP signaling patterns the intermediate and dorsal embryonic spinal cord by regulating expression of TFs in these regions (Timmer et al., 2002). BMP signaling represses Olig2 expression (Mekki-Dauriac et al., 2002); how it accomplishes this is unknown, but indirect enhancement of Irx3 expression may be involved (Meyer and Roelink, 2003), which in turn represses Olig2 expression (Novitch et al., 2001). As BMP signaling represses Olig2 expression, it should inhibit OG specification. Indeed, grafting BMP2-producing cells *in ovo* alongside ventral aspects of chick E3 trunk neural tubes strongly inhibits development of O4$^+$ cells (Mekki-Dauriac et al., 2002). In contrast, blocking BMP signaling by grafting noggin-producing cells *in ovo* alongside dorsal aspects of chick E3 trunk neural tubes results in a dorsal expansion of O4$^+$ cells in the vVZ on the grafted side (Mekki-Dauriac et al., 2002). Furthermore, BMP signaling plays a similar role in the brain since treatment of E16 rat cortical cultures with BMP2 or noggin significantly decreases or increases, respectively, the number of O4$^+$ cells (Mabie et al., 1999). Hence, BMP signaling negatively regulates OG specification; however, whether downstream effectors in the pathway vary depending on the region has yet to be determined.

Recent research shows that OPCs in the murine embryonic spinal cord can also arise from a dorsal region of the ventricular zone at later stages of development (Battiste et al., 2007; Cai et al., 2005; Vallstedt et al., 2005). Unlike the pMN domain, OG specification in this region occurs independently of Shh signaling (Cai et al., 2005). Instead, late dorsal appearance of OPCs in the spinal cord may be due to a reduction in BMP signaling with continued embryonic development (Vallstedt et al., 2005). Dorsally-derived OPCs are also seen in the hindbrain (Vallstedt et al., 2005) and telencephalon (Kessaris et al., 2006); however, whether BMP affects OG specification in these regions will require further research.

Neural-Glial Switch: Permissive Factors

In the developing spinal cord gliogenesis is preceded by neurogenesis (Zhou and Anderson 2002). For example, the pMN domain gives rise to motor neurons prior to OGs (Fu et al., 2002) (Fig. 2). During neurogenesis, Olig2 is co-expressed with the TFs Ngn1 (Zhou et al., 2001) and Ngn2 (Novitch et al., 2001; Zhou et al., 2001). Downregulation of Ngn1/2 in the pMN domain is critical to OG specification, since electroporation of chick embryonic spinal cords with Olig2 fails to promote ectopic OG

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specification unless Ngn1/2 expression is repressed, such as via Notch signaling (Zhou et al., 2001). Although electroporation with a constitutively active form of Notch and its downstream effector Suppressor of Hairless repressed Ngn1/2 expression, it did not promote ectopic OG specification unless electroporated with Olig2 (Zhou et al., 2001). Hence Notch signaling provides an environment that favors OG specification. Accordingly, when Notch signaling activity is reduced, OPCs fail to appear on schedule in zebrafish spinal cords (Park and Appel, 2003); associated changes include an excess of neurons and a loss of *olig2* expression at 1 day postfertilization (Park and Appel, 2003). A similar phenotype is seen in the spinal cords of chick embryos that undergo *in ovo* electroporation with short interfering RNAs (siRNAs) directed against the TF NFIA (Deneen et al., 2006). NFIA is required to maintain expression of Hes5 (Deneen et al., 2006), which is a downstream target in the Notch signaling pathway (Wang et al., 1998).

As Notch ligands and receptors are expressed in distinct and overlapping neuroepithelial domains in the embryonic spinal cord (Lindsell et al., 1995, 1996), Notch signaling may also play a permissive role in dorsal OG specification at later stages of development. Although multiple Notch receptors are expressed in the embryonic CNS (Lindsell et al., 1995, 1996), research examining the role of Notch signaling in OG development has primarily focused on Notch1 (Genoud et al., 2002; Givogri et al., 2002), which also inhibits neuronal differentiation (Park and Appel, 2003). How Notch1 fulfills its functions in both motor neuron and OG development is unknown but may involve Notch3, which represses Notch1-mediated transcriptional activation of Hes TFs in a concentration-dependent manner (Beatus et al., 1999). Interestingly, in the lumbar region of the E13.5 rat spinal cord Notch3 expression appears greater than Notch1 in the pMN domain (Lindsell et al., 1996), whereas the opposite appears true at E14.5 (Lindsell et al., 1995). Hence Notch3 signaling during late neurogenesis could inhibit Notch1 activity and hence allow neuronal differentiation. Prior to oligodendrogenesis a decrease of Notch3 expression in the pMN domain could restore Notch1 activity and provide an environment favoring OG specification.

Notch ligands and receptors are expressed in neuroepithelial regions of the E13.5 rat brain (Lindsell et al., 1996), supporting the view that Notch signaling may play a similar role in oligodendrogenesis throughout the CNS; however, further research is needed to verify this.

Another potential factor involved in the switch from neurogenesis to gliogenesis is the TF Sox9. Although specific ablation of Sox9 expression in nestin-positive neural stem cells does not alter Olig2, Nkx2.2, Nkx6.1, and Irx3 expression domains, there is an initial significant reduction in OPCs that recovers partially with continued embryonic development (Stolt et al., 2003). These mice also exhibit a significant transient increase in motor neurons (Stolt et al., 2003). The incomplete nature of these effects is most likely due to the presence of two additional class E Sox TFs—Sox10 and Sox8—which appear to compensate for the loss of Sox9 (Stolt et al., 2003). Therefore, Sox9, as well as potentially Sox8 and Sox10, appear to favor the switch from neurogenesis to oligodendrogenesis in the spinal cord. However, further research must determine how this is accomplished, as well as whether any links to Notch signaling exist. In addition, whether Sox9's role is similar throughout the CNS is not known.

Recent research suggests the SoxD TFs, Sox5 and Sox6, may interfere with SoxE function in the spinal cord by competing for binding sites and/or interaction partners (Stolt et al., 2006). This may explain why OPCs arise earlier and are transiently increased in the spinal cords of Sox5/Sox6 double-deficient mice. However, as the number of motor neurons in these mutant mice is not altered (Stolt et al., 2006), further research will need to clarify the exact roles of the SoxE and SoxD TFs in OG specification as well as how they relate to one another.

MATURATION OF OGs

Inhibitory Factors

Following specification, Notch signaling inhibits premature OG differentiation in the embryonic spinal cord. In particular, heat shock induced ubiquitous expression of a constitutively active form of zebrafish Notch1a following OG specification greatly reduces proteolipid protein 1 (*plp1*)/*dm20*$^+$ cells (Park and Appel, 2003). Conversely, ablation of Notch1 in oligodendroglial cells results in a dramatic increase in cells expressing high levels of PLP/DM20 mRNA in the E17.5 murine spinal cord (Genoud et al., 2002).

Notch signaling is also important in preventing premature OG maturation in the brain. In particular, activation of the Notch pathway in purified OPC cultures results in a large proportion of OPCs failing to differentiate into GalC$^+$ OGs (Wang et al., 1998). In contrast, heterozygous mice deficient in Notch1 display a transient increase in myelin basic protein (MBP) and PLP expression in numerous brain regions (Givogri et al., 2002). Furthermore, ablation of Notch1 in oligodendroglial cells results in a significant increase of perinuclear myelin-associated glycoprotein (MAG)$^+$ cells in the newborn cerebrum (Genoud et al., 2002).

Additionally, overexpression in purified OPCs of the TF Hes5, a downstream target of Notch signaling (Wang et al., 1998), results in most cells failing to differentiate (Kondo and Raff, 2000a). Hes5 appears to inhibit OG maturation via multiple mechanisms. In particular, it can directly repress Sox10 expression, which is important for OG maturation. In addition, it can repress MBP promoter activity in Oli-neu cells. Although Hes5 can bind to the MBP promoter, this binding is not required for its repression of promoter activity. Therefore, this suggests Hes5 indirectly represses MBP expression. One mechanism by which Hes5 may accomplish this is via binding to Sox10 and/or Mash1 (Liu et al., 2006). As both these TFs activate the MBP promoter in Oli-neu

cells (Gokhan et al., 2005), Hes5 could repress MBP transcription by interfering with their function. Correspondingly, Hes5 is only able to repress MBP promoter activity when Sox10 is expressed at low levels (Liu et al., 2006). Sox10 expression levels appear to increase after primary oligodendroglial cells are induced to differentiate *in vitro* (Dugas et al., 2006; Wei et al., 2004).

Retinoic acid (RA) also inhibits premature maturation since treatment of spinal cord cultures with RA results in a substantially reduced number of $O4^+$ and $GalC^+$ cells (Noll and Miller, 1994). Furthermore, differentiation of purified cerebral OPCs into $GalC^+$ OGs is attenuated by treatment with a high concentration of RA (Laeng et al., 1994). How RA accomplishes this is unknown, but it may involve its ability to enhance expression of polysialylated neural cell adhesion molecule (PSA-NCAM) (Husmann et al., 1989), which is implicated in inhibiting OG differentiation (Decker et al., 2000). In particular, RA enhances activity of sialyltransferase, which adds sialic acid residues to the NCAM protein, in a concentration-dependent manner (Deutsch and Lotan, 1983). As this effect is first evident 1 day after RA treatment (Deutsch and Lotan 1983), downstream targets of RA, such as Hox and Krox TFs (Conlon and Rossant, 1992), may be involved. Hox TFs are indeed implicated in the regulation of NCAM expression (Jones et al., 1992). Furthermore, Hoxa2 (Hao et al., 1999; Nicolay et al., 2004a) and Hoxb4 (Nicolay et al., 2004b) are expressed by cells of the OG lineage. Therefore, RA may inhibit OG maturation by enhancing expression of PSA-NCAM, potentially with the aid of downstream effectors such as *Hox* genes.

BMP signaling, which initially inhibits OG specification, subsequently inhibits OG maturation. In particular, placement of BMP4-soaked beads at the ventral midline of murine cervical spinal cord/hindbrain explants results in a dramatic decrease in PLP^+ and MAG^+ cells in the area around the beads (See et al., 2004). In addition, treatment of immunopurified cerebral OPCs, which were first allowed to mature to the $GalC^+$ stage in differentiation medium, with BMP4 severely reduces MBP and PLP expression (See et al., 2004).

BMP signaling may inhibit OG maturation by inducing expression of the TFs Id2 and Id4 (Samanta and Kessler, 2004). Indeed, overexpression of Id2 (Wang et al., 2001) or Id4 (Kondo and Raff, 2000b) in purified OPCs cultured in differentiation medium results in most cells failing to mature into $GalC^+$ OGs. One way in which Id2 and Id4 may inhibit OG maturation is by negatively regulating the function of Olig1 and Olig2, which are believed to promote maturation (Fu et al., 2002; Lu et al., 2002). In particular, Olig1 and Olig2 interact with E2A TFs (Samanta and Kessler, 2004), which are expressed by oligodendroglial cells and are believed to mediate effects of Olig TFs (Sussman et al., 2002). Id2 and Id4 are believed to interfere with Olig/E protein heterodimerization since they interact with both Olig1 and Olig2 (Samanta and Kessler, 2004) as well as E2A proteins (Sun et al., 1991). Hence, BMP signaling may inhibit OG maturation by enhancing Id TF expression, which blocks Olig1 and Olig2 function.

Sox5 and Sox6 are also important for inhibiting OG maturation since a significant increase in MBP- and PLP-immunoreactive cells is apparent in the spinal cords of 18.5 days post coitum (dpc) Sox5/Sox6 conditional-double deficient mice (Stolt et al., 2006). These TFs appear to inhibit OG maturation by interfering with Sox10 function. In particular, Sox5 and Sox6 can block Sox10-directed activation of the MBP promoter in oligodendroglial cell lines. These TFs may block Sox10 function via multiple mechanisms as they can recognize Sox10-binding sites and interaction partners (Stolt et al., 2006).

Promoting Factors

Once OPCs reach their final destination, maturation ensues. Various TFs, including Olig1, Olig2, Nkx2.2, Nkx6.2, and MyTI, favor OG maturation. For example, in Olig1 mutant mice, PLP/DM20 and MBP expression is markedly reduced in postnatal day 0 (P0) (Lu et al., 2002) and P14 (Xin et al., 2005) spinal cord and undetectable in the P14 corpus callosum, neocortex, and striatum (Xin et al., 2005). Similarly, the number of $GalC^+$ and PLP^+ OGs is significantly reduced in spinal cord cultures following Olig2 antisense treatment (Fu et al., 2002). How Olig1 and Olig2 promote maturation is unknown but may involve their regulation of specific TFs. One such TF is Zfp488, which is a potential downstream target of Olig1; Zfp488 expression is lost in the spinal cord, optic nerves, cerebellum, and corpus callosum of Olig1 mutant mice (Wang et al., 2006). Downregulating *Zfp488* expression in an oligodendroglial cell line results in a reduction in *MBP* and 2′,3′-cyclic nucleotide 3′-phosphodiesterase (*CNP*) expression. Interestingly, Zfp488's transcription appears to be specifically regulated by Olig1 since only Olig1, but not Olig2, can significantly enhance luciferase reporter activity driven by its putative promoter. Olig2 appears to interact with Zfp488 at the protein level (Wang et al., 2006); however, further research must determine if this interaction is required for their function.

Ectopic expression of Olig2 in chick embryonic spinal cords via *in ovo* electroporation induces Sox10 expression (Liu et al., 2007). Furthermore, Sox10 expression is undetectable throughout the spinal cord in the absence of Olig2 (Lu et al., 2002). Olig1, whose expression reappears after Sox10 in the rodent embryonic spinal cord (Zhou et al., 2000), may be important in maintenance of Sox10 expression since Olig1 mutant mice exhibit a decrease in $Sox10^+$ cells (Lu et al., 2002; Xin et al., 2005). As researchers have yet to find a chick Olig1 ortholog (Zhou et al., 2001), this may explain why strong expression of Sox10 is seen much earlier in rodents (Zhou et al., 2000) than in the chick (Zhou et al., 2001).

Sox10 is important in OG maturation since a delay and a dramatic decrease in the expression of PLP and MBP occurs in the spinal cords of Sox10-deficient mice (Stolt et al., 2002). Furthermore, transplantation of Sox10-deficient neural stem cells into 3–9 day old mouse

retinas failed to myelinate the host nerve fiber layer (Stolt et al., 2002). More recently, Dugas et al. (2006) used siRNA to prevent normal induction of Sox10 expression in OPCs *in vitro* and found a significant reduction in cells differentiating into MBP-expressing cells. In addition, overexpressing Sox10 in embryonic chick spinal cords via *in ovo* electroporation induces expression of Nkx2.2, Olig2, MBP, PLP, and MAG (Liu et al., 2007). Some MAG^+ cells co-express Nkx2.2 or Olig2 (Liu et al., 2007), which suggests Sox10 may indirectly enhance myelin protein gene expression through these TFs. However, as a proportion of the MAG^+ cells do not express Nkx2.2 or Olig2 (Liu et al., 2007), Sox10 may also directly induce myelin protein gene expression. Indeed, Sox10 appears to directly regulate MBP expression since it can bind to three distinct sites in MBP's proximal promoter. Accordingly, Sox10-directed activation of the MBP proximal promoter is reduced or almost completely blocked by mutating the three sites individually or all together, respectively (Stolt et al., 2002).

Sox10 also appears to regulate transcription of two gap junction proteins—connexin47 (Schlierf et al., 2006) and connexin32 (Bondurand et al., 2001; Schlierf et al., 2006)—which are important for normal CNS myelination (Menichella et al., 2003). In particular, Sox10 activates the connexin32 promoter (Bondurand et al., 2001; Schlierf et al., 2006) as well as the connexin47 promoter 1b (Schlierf et al., 2006) in luciferase reporter gene assays. This activation appears to be direct since it is dramatically reduced by mutations in either the Sox10 DNA-binding domain or binding sites (Bondurand et al., 2001; Schlierf et al., 2006). These connexin proteins are important for normal CNS myelination since connexin32/connexin47 double knockout mice exhibit profound CNS myelin abnormalities, such as thin, vacuolated, or absent myelin sheaths (Menichella et al., 2003).

Since Sox10 is a poor transcriptional effector on its own (Kuhlbrodt et al., 1998b), other TFs are likely implicated in this process. One potential TF is SCIP, which is expressed by oligodendroglial cells (Collarini et al., 1992) and functions synergistically with Sox10 (Kuhlbrodt et al., 1998b). Surprisingly, myelination occurs normally in the spinal cords of SCIP mutant mice (Bermingham et al., 1996). In contrast, mice lacking the ligand-dependent TF COUP-TFI, which is a potential upstream regulator of SCIP, exhibit CNS hypomyelination and dysmyelination (Yamaguchi et al., 2004). The reason for the discrepancy in mutant phenotypes is unknown but may be due expression of two additional class III POU TFs, Brn-1 and Brn-2, which could potentially be regulated by COUP-TFI in oligodendroglial cells (Schreiber et al., 1997) and hence could compensate for SCIP in its absence. Also, oligodendroglial cells express two additional Sox proteins, Sox4 and Sox11, which function synergistically with Brn-1 and/or Brn-2 (Kuhlbrodt et al., 1998a).

Other TFs that function synergistically with Sox10 are Olig2 and Sp1. In particular, when Olig2 and its heterodimerization partner E47 are co-transfected with Sox10 in Oli-neu cells, a synergistic increase in MBP promoter activity occurs (Gokhan et al., 2005). As Sox10 can interact with Olig2 (Stolt et al., 2006), this synergistic activation may be dependent upon formation of a transcriptional complex. Like Olig2, a synergistic activation of the MBP promoter is also seen when a mouse embryonic fibroblast cell line NIH 3T3 is co-transfected with Sox10 and the TF Sp1 (Wei et al., 2004); however, whether these TFs interact at the protein level has yet to be determined.

Sox8, another group E Sox TF, also plays a role in OG maturation since a transient reduction occurs in the number of MBP^+ and PLP^+ cells in the spinal cords of Sox8-deficient mice (Stolt et al., 2004). Hence myelin protein expression seen in Sox10-deficient mice may be due to compensatory effects of Sox8 and vice versa. In fact, Sox8 has a limited ability to compensate for Sox10 since a dramatic decrease in *MBP* and *PLP* expression occurs in the spinal cords of P7 transgenic mice, in which Sox10 is replaced by Sox8 (Kellerer et al., 2006).

In addition to Sox10, Olig2 also appears to regulate expression of Nkx2.2. In particular, ectopic Nkx2.2 expression is found 5 days following *in ovo* electroporation of E2 chick spinal cords with an Olig2-expressing vector (Liu et al., 2007). Evidence supporting Nkx2.2's role in promoting OG maturation is the finding that in its absence, a delay and a dramatic decrease in PLP/DM20 and MBP expression occurs in the spinal cord (Qi et al., 2001). Furthermore, in Nkx2.2 mutant mice MBP expression is reduced or lost in the E17.5 medulla and P7 forebrain, respectively (Qi et al., 2001). Correspondingly, Ngn3 mutant mice, which lack Nkx2.2 expression in the E13.5 spinal cord, display a dramatic reduction in PLP and MBP expression at P0 (Lee et al., 2003).

How Nkx2.2 promotes OG maturation is uncertain, but it appears to involve its ability to regulate expression of certain myelin protein genes. In particular, overexpressing Nkx2.2 in NIH 3T3 cells can induce green fluorescent protein (GFP) expression driven by a portion of the PLP promoter (Qi et al., 2001). This induction may be direct since consensus Nkx2.2-binding sites are found in the PLP promoter (Qi et al., 2001). Interestingly, MBP promoter also contains Nkx2.2-binding sites (Qi et al., 2001). In contrast to PLP, however, Nkx2.2 represses MBP promoter activity either on its own (Wei et al., 2005) or directed by certain other TFs (Gokhan et al., 2005). Although these results appear to contradict the transgenic knockout data, they may reflect a cell line- or CNS region-specific effect. In particular, these transient transfection assays were conducted utilizing two oligodendroglial cell lines—CG4 and Oli-neu (Gokhan et al., 2005)—established from primary oligodendroglial cultures from either perinatal rat cerebral cortex (Louis et al., 1992) or E15 murine brains (Jung et al., 1995), respectively. Interestingly, Nkx2.2's expression profile during OG development varies not only between these two cell lines, but also with primary cultures obtained from the embryonic murine spinal cord (Fu et al., 2002; Gokhan et al., 2005; Qi et al., 2001; Wei et al., 2005). Hence, further research is needed to resolve this issue.

Another Nkx TF, Nkx6.2, exhibits an expression profile similar to MBP and PLP (Awatramani et al., 1997). Although Nkx6.2 has multiple binding sites within the PLP and MBP promoters (Awatramani et al., 1997), MBP is similarly expressed in the spinal cord, forebrain, and cerebellum of wild-type and Nkx6.2 mutant mice (Cai et al., 2001). However, Nkx6.2 is required for normal myelin ultrastructure (Southwood et al., 2004).

The TF MyTI may also promote OG maturation since co-transfection of oligodendroglial cells with a plasmid expressing the seven zinc-finger isoform of MyTI and a CNP-luciferase reporter results in a 6.7-fold induction in reporter activity when compared with the empty vector (Nielsen et al., 2004). Furthermore, infection of rat OPC cultures with a retrovirus containing a truncated MyTI, which is expected to interfere with endogeneous MyTI function, results in a significant reduction in the differentiation of oligodendroglial cells (Nielsen et al., 2004).

Sox17, a Sox F TF, also appears important in OG maturation (Sohn et al., 2006). In particular, down-regulation of Sox17 expression in purified OPCs with siRNAs results in a significant increase or decrease in the percentage of $A2B5^+$ and $O4^+$ cells, respectively. Not only are these findings reversed in OPCs, which overexpress Sox17, but these cells also display increased RNA levels of MBP, MAG, and CNP. Sox17 may promote maturation via several mechanisms. For instance, overexpression of Sox17 in purified OPCs results in a significant decrease in the percentage of proliferative cells, which suggests it could promote cell cycle exit. In addition, Sox17 may regulate transcription of particular myelin protein genes; for instance, it can enhance luciferase activity driven by the MBP promoter (Sohn et al., 2006). However, whether this effect is direct or indirect is not known and will require further research. Surprisingly, Notch signaling also promotes OG maturation (Cui et al., 2004; Hu et al., 2003). Although unexpected given the previous information, this could reflect a ligand-, receptor-, and/or downstream effector-dependent event. Indeed, evidence suggests it could be ligand-dependent; whereas activation of Notch signaling via the standard Notch ligands, Jagged1 or Delta1, results in most OPCs failing to differentiate (Hu et al., 2003; Wang et al., 1998), treatment of purified OPCs with the novel Notch ligands, F3 or NB-3, promotes their maturation into CNP^+ OGs (Cui et al., 2004; Hu et al., 2003). In contrast, current data do not support a receptor-dependent effect since Notch1 appears important for inhibiting (Genoud et al., 2002; Givogri et al., 2002) as well as promoting (Cui et al., 2004; Hu et al., 2003) OG maturation. However, as Notch2 and/or Notch3 are expressed in similar areas as Notch1 in the embryonic and postnatal CNS (Irvin et al., 2001; Lindsell et al., 1996), further research must clarify which receptors are involved in each process. In comparison, some evidence supports a downstream effector-dependent event. In particular, the Delta1/Notch signaling pathway in OPCs appears to utilize CBF1 (Wang et al., 1998), whereas the F3,NB-3/Notch signaling pathway in the same cells requires Deltex1 (Cui et al., 2004; Hu et al., 2003). Interestingly, these Notch signaling pathways also appear to activate distinct target genes since exposing oligodendroglial cells to Delta1 or F3/NB-3 enhances mRNA expression of Hes5 (Wang et al., 1998) or MAG (Cui et al., 2004; Hu et al., 2003), respectively. Hence, various factors may dictate the effect of Notch signaling on OG maturation.

Researchers have also found treatment of either primary mixed glial cultures or purified OPCs with RA promotes OG maturation (Barres et al., 1994; Givogri et al., 2001; Pombo et al., 1999). The obvious disparity between these findings and those in the previous section is likely due to discrepancies in experimental protocols utilized by individual research groups. In particular, although all conducted *in vitro* culture assays, each research group utilized different ages, CNS regions, and/or RA concentrations (Barres et al., 1994; Givogri et al., 2001; Laeng et al., 1994; Noll and Miller, 1994; Pombo et al., 1999). As such, the contradictory results may be due to a stage- and/or concentration-dependent effect(s). Current research favors the former since treatment of newborn cerebral cultures with micromolar concentrations of RA impairs OG maturation if they are first purified for OPCs (Laeng et al., 1994), whereas it promotes this process if the cells are grown in culture for 1 week prior to treatment (Givogri et al., 2001). In contrast, current data do not support a concentration-dependent effect since both micromolar and nanomolar concentrations of RA promote OG maturation *in vitro* (Barres et al., 1994; Givogri et al., 2001; Pombo et al., 1999). However, as these data were obtained using different experimental protocols, further research must also resolve this issue.

RA exists as two distinct isomers—all-*trans* and 9-*cis*—which can differentially bind to specific receptors. In particular, 9-*cis* RA can bind to both retinoic acid receptors (RARs) and retinoid X receptors (RXRs), whereas all-*trans* RA can only bind to the former (Allenby et al., 1993). These receptors, which exist as multiple isoforms, bind primarily as heterodimers to RA response elements found in the regulatory regions of various genes (Maden, 2002). Intriguingly, evidence suggests RA's effect on OG maturation could be receptor-dependent. In particular, although all-*trans* RA enhances *MBP* expression (Givogri et al., 2001), it is unable to activate the MBP promoter when RARα is transfected alone or in combination with RXRα (Pombo et al., 1999). Since the isomer dictates which receptors are bound, it may also affect the outcome. Although most groups utilized all-*trans* RA (Barres et al., 1994; Givogri et al., 2001; Laeng et al., 1994; Noll and Miller, 1994), the extent of isomerization in the cultures, as well as any stage-dependence, is unknown; further research is required to resolve this issue.

RA may promote OG maturation via several mechanisms. For instance, RAR can repress the promoter activity of the TF E2F-1, which induces expression of genes involved in DNA replication or mitosis (Polager et al., 2002) in the presence of RA (Nygärd et al., 2003). This repression, in turn, could lead to cell cycle withdrawal and subsequent OG maturation. In addition, RA's ability to promote maturation depends upon mem-

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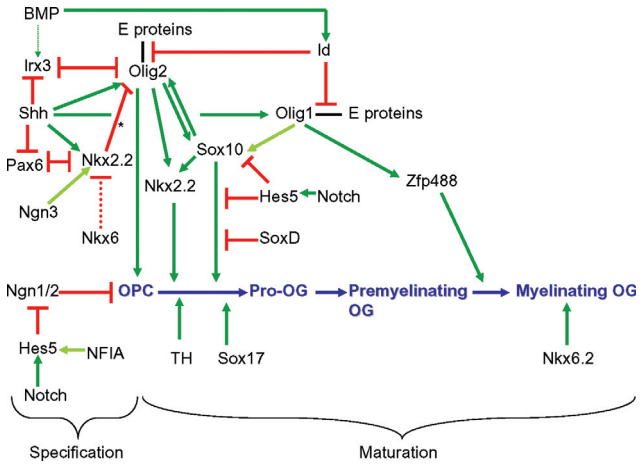

COLOR

Fig. 3. Basic transcriptional network of OG development. For simplicity reasons the figure depicts transcriptional control of oligodendroglial cells arising from the pMN domain in the rodent embryonic spinal cord. *Specification:* Many TFs (e.g., Olig2, Nkx2.2, Pax6) and signaling pathways (BMP, Notch, Shh) are important for OG specification in the pMN domain. Many of these factors are involved in the dorsoventral patterning of the spinal cord (upper left). For instance, Shh directs formation of five ventral progenitor domains, including the pMN domain, by regulating expression of TFs in the vVZ. This includes regulating expression of Nkx2.2 and Pax6, as well as Olig2 and Irx3, which define the ventral and dorsal boundaries, respectively, of the pMN domain. Subsequently, cross-repressive interactions between these TFs maintain and refine these boundaries. Of these TFs, only Olig2 is critical for OG specification in this region; however, it cannot promote OG development unless Ngn1/2 expression is downregulated such as via Notch signaling (bottom left). *Maturation:* Several TFs (e.g., Olig2, Sox10, Olig1, Hes5) and signaling pathways (BMP, Notch) affect OG maturation. Most of these factors appear to act, in part, by modulating expression and/or function of other factors. For example, Olig1 may promote OG maturation by maintaining or inducing expression of Sox10 and Zfp488, respectively. In contrast, Hes5 may impair this process by inhibiting expression and/or function of Sox10, which promotes OG maturation. For more information on individual factors please refer to the text. (⊥), inhibit/repress; (→), promote/induce; (→), maintain; (—), heterodimers; (dotted line), indirect effect; Id, Id2/Id4; Nkx6, Nkx6.1/6.2; TH, thyroid hormone; *,effect is only evident during early stages of development.

bers of the p53 TF family since RA-induced maturation is blocked when purified OPCs are infected with dominant-negative forms of p53 or p73 (Billon et al., 2004). How p53 and p73 promote OG maturation is uncertain, but it may involve their ability to induce gene expression of two cyclin-dependent kinase inhibitors—p21 and p27 (Fontemaggi et al., 2002). Overexpressing p27 in CG4 cells enhances MBP promoter activity (Wei et al., 2003, 2004) by increasing Sp1 binding to the MBP promoter (Wei et al., 2003). Accordingly, a dramatic decrease in MBP expression is seen in the cerebellum of p21−/− mice at P4 and P6 (Zezula et al., 2001).

Thyroid hormone (TH) also promotes OG differentiation; treatment of E14.5 murine spinal cord explants with the TH triiodothyronine (T_3) increases both MBP^+ cells and the formation of structures resembling myelin internodes (Park et al., 2001). T_3 has multiple stimulatory effects on the expression of several myelin protein genes in aggregating telencephalic cultures (Tosic et al., 1992). The importance of TH in OG maturation in the brain is evident from studies utilizing hypothyroid and hyperthyroid animals. In particular, hypothyroid rats exhibit a transient reduction in expression levels of various myelin protein genes (Ibarrola and Rodríguez-Pe~na, 1997). Conversely, rats treated with the TH thyroxine (T_4) exhibit higher MBP and PLP mRNA levels (Ibarrola and Rodríguez-Pe~na, 1997). The effects of TH on OG maturation appear to be mediated by the TH receptor alpha 1, which acts as a ligand-dependent TF, since in its absence a significant decrease in the percentage of $GalC^+$ OGs in TH-treated optic nerve cultures is found (Billon et al., 2002). The mechanisms by which TH promotes maturation appear similar to those of RA (Billon et al., 2004; Nygard et al., 2003).

CONCLUSIONS

Over the past decade enormous strides have been made in our understanding of the transcriptional control of OG specification, particularly in the pMN domain. In this region OG specification is largely the result of interplay between Shh, BMP, and Notch signaling pathways, which regulate expression of TFs dictating spatial and/or temporal aspects of oligodendrogenesis (Fig. 3). F3 Although much less is known regarding regulation of this process in other regions, several similarities and differences have been identified. However, as most of this information was obtained prior to the discovery of distinct ventral and dorsal OPC progenitor domains in the spinal cord (Cai et al., 2005; Vallstedt et al., 2005), hindbrain (Vallstedt et al., 2005), and telencephalon (Kessaris et al., 2006), it must be re-examined with this in mind. Complicating this research is the expression of similar oligodendroglial markers by both dorsal and ventral progenitors, making it difficult to distinguish between the two (Cai et al., 2005; Kessaris et al., 2006; Vallstedt et al., 2005). This will be particularly problematic with respect to dorsal OPCs since they often arise after their ventral counterparts (Cai et al., 2005; Kessaris et al., 2006). A redeeming fact, evident from knowledge of transcriptional control of OG specification in the pMN domain, is that signaling pathways and TFs involved in dorsoventral patterning of a particular progenitor domain will likely affect OG specification in that region. As information regarding this process is available for many CNS areas, it will greatly expedite research into transcriptional control of OG specification in distinct progenitor domains.

In addition to specification, researchers have made huge contributions to understanding transcriptional control of OG maturation. In most cases, TFs and signaling pathways identified either inhibit or promote this process; however, instances exist where the effect of a particular signaling pathway appears dependent upon such factors as ligand, receptor, and/or stage of oligodendrogenesis. Intriguingly, many signaling pathways and TFs appear to act, in part, by affecting expression and/or function of other TFs (Fig. 3). In addition, many TFs and signaling pathways appear to play similar roles throughout the CNS. However, since some of this information was obtained utilizing *in vitro* assays (Barres et al., 1994; Cui et al., 2004; Hu et al., 2003; Laeng

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TABLE 2. Expression profiles of PDGFαR (♣), PLP/DM20 (♦), and TFs implicated in OG development in the CNS

	Telencephalon		Diencephalon			Rhombencephalon		
Age	Cortex/fiber tracts	Ganglionic eminences/basal ganglia	Thalamus	Hypothalamus	Mesen-cephalon	Cerebellum	Medulla/pons	Spinal cord
E12-12.5	Id2[20],4[9] POU[8] SoxC[6]	Mash1[6] Olig2[33] POU[2] Sox11[6]	Mash1[15] Nkx2.2[23] ♣[24] POU[2]	Ngn3[27] Nkx2.2[23] ♣[24] POU[2]	Id2[20],4[9] POU[8] Sox11[12]	Id2[20]	Brn-1[8] Id2[20],4[9] Ngn3[27] Nkx2.2[23] Olig2[37] ♣[24] Sox11[12]	Hoxa2[21],b4[22] Id4[9] Mash1[15],Ngn3[27] Nkx2.2[21] Olig1,2[41] ♣[24] POU[8] SoxC[12,6],D[29],E[41,30,31]
E13-13.5	Hes5[5] Hoxa2[39] SCIP[32]	Hes5[5] Hoxa2[39] Mash1[33] Olig1,2[19,33] ♣[34],♦[35] POU[2]	Hoxa2[39] Mash1[33] Nkx2.2[23] Olig1,2[41] ♦[19,35] POU[2]	Hoxa2[39] Mash1[33] Ngn3[27] Nkx2.2[23] Olig1,2[33] ♦[19,35] POU[2]	Hes5[5] Mash1[33] SCIP[32] Sox11[12]	Hes5[5] SCIP[32]	Hes5[5] Ngn3[27] Nkx2.2[37] Olig1,2[41] ♣[24],♦[35] SCIP[32] Sox10[13],11[12]	Hes5[5] Hoxa2[7] Ngn3[14,27] Nkx2.2[26] Olig1,2[18,41] ♣[18,41,24] Sox9[31],10[41],11[12] Sox17[28]
E14-14.5	Hes5[1] SoxC[6]	Olig1,2[34] ♣[34] POU[2] Sox10[34]	Hes5[1] Hoxa2[39] Olig1,2[41] ♣[24] POU[2] SoxC[6]	Hes5[1] Ngn3[27] Olig1,2[34] ♣[34],♦[35] POU[2] Sox10[34]	Hes5[1] Sox11[12]	Hes5[1]	Hes5[1] Ngn3[27] Olig1,2[41] Sox11[12]	Hes5[1] Hoxa2[21],b4[22] Ngn3[27,14] Nkx2.2[18,21] Olig1,2[41] ♣[18,41],♦[38] SoxC[12,6],D[29],E[41,30,31] Zfp488[38]
E15-15.5	Id2[20] POU[8] Sox11[12]	Olig2[19] ♣[19] Sox11[12]	Nkx2.2[23] ♦[35] Sox11[12]	Ngn3[27] Nkx2.2[23] ♦[35] Sox11[12]	♣[24] POU[8,32] Sox11[12]		Ngn3[27] ♣[24],♦[35] POU[8,32] Sox10[13],11[12]	Brn-1,2[8] Hoxa2[7] Ngn3[27] Nkx2.2[26] Olig1,2[18,33] ♣[18,41,24],♦[18,35] SoxE[41,30,31],11[12]
E16-16.5	♣[25]	POU[2]	♣[25] POU[2]	Ngn3[27] ♣[25],♦[35] POU[2]			♣[25],♦[35] Sox10[40]	Hoxa2[21],b4[22] Nkx2.2[21] Olig2[21] ♦[35] Sox8[30],9[31],17[28], D[29]
E17-17.5	Brn-2[8],SCIP[32] Hes5[1] Id2[20] ♣[34] Sox10[13],C[12,6]	Brn-2[8] ♣[34]	Hes5[1] SoxC[12,6]	Brn-2[8] Ngn3[27] ♦[35] Sox11[12]	SCIP[32] Sox11[12]	Id2[20] Olig2[26] ♣[26] Sox11[12]	Hes5[1] Olig2[26] ♣[26],♦[35] Sox11[12]	Hes5[1] Hoxa2[7] ♣[24],♦[35] Sox9[32],C[6]
E18-18.5	Olig1,2[34] ♣[34] rKr2[16] SCIP[32] Sox10[34]			Nkx6.2[10] ♦[35]	Nkx6.2[10] SCIP[32]		Nkx6.2[10] ♦[35]	Hoxa2[21],b4[22] Nkx2.2[21],6.2[10] Olig2[21] ♣[26],♦[26,35] SoxD[29],E[4,30,31] Zfp488[38]
E19-19.5	Sox11[12]			♦[35]	Sox11[12]	Sox11[12]	♦[35]	Hoxa2[7] ♦[35]
PN	Brn-2,SCIP[8] Id2[20,36] MyTI[3], rKr2[16] Olig1,2[33] ♣[25],♦[26,35] SoxE[13],C[6], 17[28] Zfp488[38]	♣[25]	♣[25]	♣[25],♦[35]	♣[25]	Id2[20,36] Nkx6.2[10] Olig1,2[33] ♣[25],♦[35] SoxC[6],E[11] Zfp488[38]	♣[25],♦[35]	Nkx2.2[26] ♣[17],♦[26,35] SoxD[29],8[30],10[17], 17[28] Zfp488[38]

POU, Brn-1,2 and SCIP; SoxC, Sox4 and 11; SoxE, Sox8, 9, and 10; PN, postnatal day (primarily P0-14).
Although TFs and/or OG markers are expressed in the same CNS region, the expression profiles may or may not overlap. For the purpose of this table information was standardized utilizing the Carnegie/Theiler staging system. Utilizing this staging system the day of vaginal plug is considered E1; therefore, the days were adjusted accordingly so as to have consistent expression data. In cases where the staging information was not given[1,4,10,16,17,20,25,26,29-31,37,38,41] the indicated age in the manuscript was utilized. The embryonic age listed is for mice; the corresponding rat embryonic age is 1.5 days older. Most references utilized *in situ* hybridization, whereas some references also utilized immunohistochemistry[3,4,7,14,15,16,21,22,28-31,33,37,39].
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et al., 1994; Nielsen et al., 2004; Noll and Miller, 1994; See et al., 2004; Sohn et al., 2006), *in vivo* verification is needed. In addition, further research must clarify where particular TFs play a role in maturation since in most cases researchers have only examined expression of myelin proteins. Furthermore, how transcriptional control of OG maturation compares between ventral and dorsal progenitors remains to be determined. Although this is a huge undertaking, it will be aided by the vast knowledge already accumulated regarding expression of

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T2 TFs in the CNS (Table 2). The importance of obtaining this information is evident from the findings that although ventral and dorsal OPCs appear able to compensate for each other if one progenitor type is ablated (Cai et al., 2005; Kessaris et al., 2006), the compensation appears limited if OPCs are present but their maturation is defective (Yue et al., 2006). Ultimately, identifying molecular mechanisms regulating OG development will be critical to establishing therapeutic strategies for demyelinating diseases, such as multiple sclerosis.

ACKNOWLEDGMENTS

DJN was supported by a CIHR Health Research Fellowship. We are grateful to Drs. Edward Hawes and Phyllis Paterson for their critique of the manuscript, and thank Dr. Mark Noble for his helpful suggestions regarding Figure 3.

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Cellular and Molecular Neurobiology, Vol. 26, Nos. 4–6, July/August 2006 (© 2006)
DOI: 10.1007/s10571-006-9058-4

Transcriptional Regulation of Neurogenesis in the Olfactory Epithelium

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Received December 31, 2005; accepted March 14, 2006; Published online: May 18, 2006

SUMMARY

1. The olfactory epithelium (OE) is a simple structure that gives rise to olfactory sensory neurons (OSNs) throughout life.

2. Numerous transcription factors (TFs) are expressed in regions of the OE which contain progenitor cells and OSNs. The function of some of these TFs in OSN development has been elucidated with the aide of transgenic knockout mice.

3. We review here the current state of knowledge on the role of TFs in OE neurogenesis and relate the expression of these TFs, where possible, to the well-documented phenotype of the cells as they progress through the OSN lineage from progenitor cells to mature neurons.

KEY WORDS: Dlx5; Hes; KLF7; Lhx2; Mash1; Mecp2; NeuroD; Ngn; Olig2; O/E; Runx1; Wt1.

INTRODUCTION

The olfactory epithelium (OE) and its underlying lamina propria comprise the olfactory mucosa, which lines the posterior/superior region of the nasal cavity (Frisch,

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Abbreviations: ACIII, adenylyl cyclase III; Atf5, activating transcription factor 5; BrdU, bromodeoxyuridine; Dlx5, *Distal-less* homeobox homolog 5; E, embryonic day; GAP43, 43 kDa growth associated protein; GBCs, globose basal cells; Golf, olfactory G protein; HBCs, horizontal basal cells; Hes, mammalian homolog of *hairy* and *Enhancer of split*; HLH, helix–loop–helix; Id, inhibitor of DNA binding; INPs, immediate neuronal precursors; KLF7, Krüppel-like factor 7; Lhx2, LIM homeobox protein 2; Mash1, mammalian *achaete-scute* homolog 1; Mecp2, methyl-CpG binding protein 2; Msx1, *msh* (muscle specific homeobox) homolog 1; NCAM, neural cell adhesion molecule; NeuroD, Neurogenic differentiation; NFI, nuclear factor I; Ngn, Neurogenin; OBs, olfactory bulbs; OcNc, olfactory cyclic nucleotide-gated channel; OE, olfactory epithelium; O/E, Olf (olfactory neuronal transcription factor)/Ebf (early B cell transcription factor); OMP, olfactory marker protein; OP, olfactory placode; ORs, odorant receptors; OSNs, olfactory sensory neurons; Otx, vertebrate homeobox gene related to *orthodenticle* (*otd*); Pax6, Paired homeobox gene 6; Roaz, rat O/E-1-associated zinc finger; Runx1, runt-related transcription factor 1; TFs, transcription factors; Wt1, Wilms' tumor gene.

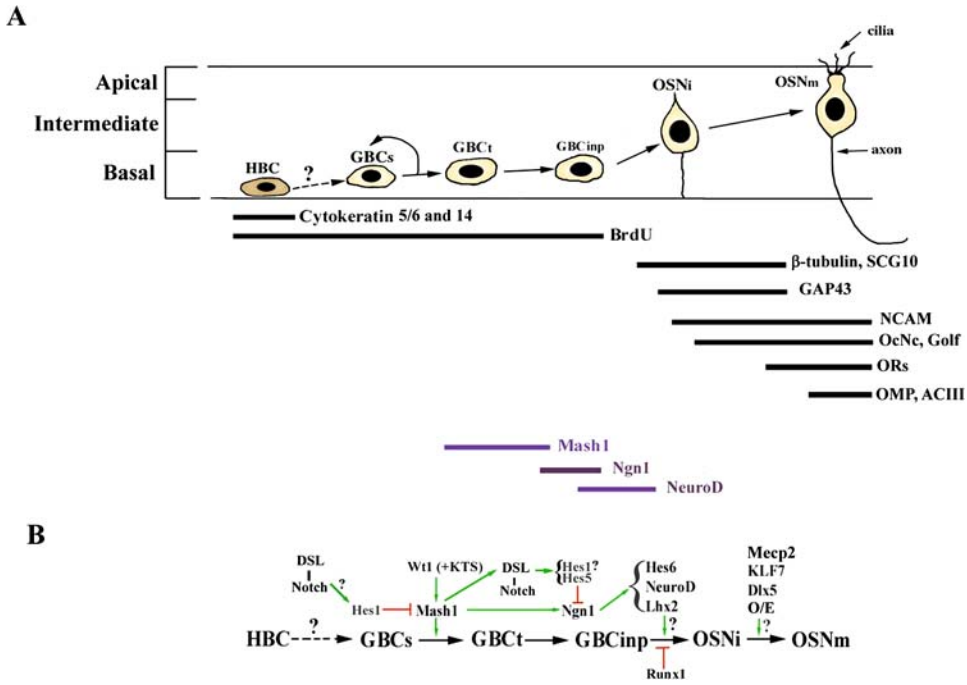

Fig. 1. Neurogenesis in the OE (A). Three distinct cell types (HBCs, GBCs, and OSNs) are believed to comprise the OSN cell lineage. These cell types can be distinguished based on morphological and antigenic phenotypes, as well as, on their location in the OE with respect to the basal lamina. Currently, there are only three TFs (Mash1, Ngn1, NeuroD; mauve-colored font) that have been studied well enough to state which cells in the OSN lineage express each TF. (B) model of transcriptional regulation of neurogenesis in the OE. See text for discussion. →: enhance/promote; ⊥: repress/inhibit; ?: uncertain. *Abbreviations:* ACIII—adenylyl cyclase type III; BrdU—bromodeoxyuridine; DSL—Delta, Serrate, and LAG-2 family of Notch ligands; GAP43—43 kDa growth associated protein; GBCs—stem cell globose basal cell; GBCt—transit amplifying globose basal cell; GBCinp—immediate neuronal precursor globose basal cell; Golf—olfactory G protein; HBC—horizontal basal cell; NCAM-neural cell adhesion molecule; OcNc—olfactory cyclic nucleotide gated channel; OE—Olfactory Epithelium; OMP-olfactory marker protein; ORs—odorant receptors; OSNi—immature olfactory sensory neuron; OSNm—mature olfactory sensory neuron.

1967; Morrison and Costanzo, 1992; reviewed in Schwob, 2002). The olfactory sensory neurons (OSNs), which detect odors in the air we breathe, are located within this epithelium (Fig. 1(A)) and continue to be generated throughout life (Graziadei and Monti Graziadei, 1979; Caggiano *et al.*, 1994; reviewed in Schwob, 2002). This lifelong neurogenesis occurs as a result of the continual proliferation and differentiation of stem/progenitor cells located near the base of the epithelium. Although some of these precursor cells seem fated to the neuronal lineage, others are multipotent and can differentiate into neuronal as well as non-neuronal cells (Schwob *et al.*, 1994; reviewed in Schwob, 2002). In addition to playing a role in neurogenesis that occurs on a daily basis, the stem/progenitor cells of the OE are also responsible for replacing OSNs that are invariably lost when the olfactory axons are injured (reviewed in Schwob, 2002).

The OE arises from a thickening of the surface ectoderm called the olfactory placode (OP), which is first evident in the mouse at embryonic day 9 (E9) and which subsequently invaginates to form the olfactory pit (Cuschieri and Bannister, 1975). The lining of the OP thickens and subsequently gives rise to three layers characteristic of the adult OE (Fig. 1(A)) (Graziadei and Monti Graziadei, 1979). The inner basal layer, which is adjacent to the basal lamina, is composed of two cell types, the horizontal (HBC) and globose (GBC) basal cells (Graziadei and Monti Graziadei, 1979). The intermediate layer contains neurons exhibiting a basal to apical maturation gradient such that mature OSNs are found closer to the outer apical layer. The apical layer also contains the nuclei and bulk of the soma of the supportive sustentacular cells (Graziadei and Monti Graziadei, 1979). At E11 in the mouse, over 95% of mitotic cells are found apically in the developing OE whereas postnatally cell proliferation and thus the generation of neuronal progenitor cells is found predominantly near the base of the OE (Smart, 1971).

Over the past decade knowledge regarding the transcriptional control of neurogenesis in the OE has increased significantly (Fig. 1 and Table 1). Although there are a large number of transcription factors (TFs) that are expressed in the OE (Table 1), expression profiles during OSN development have only been determined for a few TFs. (Fig. 1(A)). The onset of expression of helix–loop–helix (HLH) TFs during rodent OE neurogenesis appears to be similar during embryonic and neonatal development, as well as, during regeneration following methyl bromide-induced lesions in the OE of adult rats (Cau *et al.*, 1997, 2002; Manglapus *et al.*, 2004). This suggests that the transcriptional control of OE neurogenesis is consistent throughout life. Hence the OE is an excellent model from studying neurogenesis because it produces neurons throughout life and exhibits an enormous regenerative capacity (Schwartz Levey *et al.*, 1991; Caggiano *et al.*, 1994; Schwob *et al.*, 1994, 1995). Furthermore, since neurological diseases, such as Alzheimer's disease and Rett's syndrome, exhibit abnormalities in the OE (Ronnett *et al.*, 2003; Ghanbari *et al.*, 2004), analysis and culturing of OE biopsy specimens from these patients may be very useful in determining the etiology, pathogenesis, and treatment of these diseases.

This review describes what is known about the transcriptional control of neurogenesis in the OE and relates the expression of these TFs, where possible, to the well-documented phenotype of the cells as they progress through the OSN lineage from progenitor cells to mature neurons (Fig. 1, Table 1).

OSN LINEAGE: FROM PROGENITOR CELL TO MATURE NEURON

Postnatally and continuing into adulthood, three cell types (HBCs, GBCs, and OSNs) comprise the OSN lineage within the pseudostratified OE (Fig. 1(A)). HBCs and GBCs are located in the inner basal OE layer, which is adjacent to the basal lamina (Graziadei and Monti Graziadei, 1979). The HBCs, which lie directly against the basal lamina, are morphologically flat cells that express cytokeratin 5/6 and 14 (Fig. 1(A)) (Graziadei and Monti Graziadei, 1979; Holbrook *et al.*, 1995). In

Table 1. TF Expression Profiles and Function in the OE

Family	Class/group	TF	Expression profiles			Function	References
			In OE	Age	Co-expression		
ATF		Atf5	Intermediate	Embryonic, Postnatal			Hansen *et al.*, 2002
Fox	Foxa	Foxa2	Not specified	Adult			Besnard *et al.*, 2004
	Foxg	Foxg1/BF-1	N^{t}	E			Tao and Lai, 1992
HLH	II	Mash1	Basal, I, Apical^{rt}	E, P, A	BrdU, Ngn1	Development^{rt}	Cau *et al.*, 1997, 2002; Gordon *et al.*, 1995, Guillemot *et al.*, 1993; Murray *et al.*, 2003; Saito *et al.*, 1996; Manglapus *et al.*, 2004
		NeuroD	B	E, P, A	β-Tubulin, BrdU		Cau *et al.*, 1997, 2002; Suzuki *et al.*, 2003a; Manglapus *et al.*, 2004
		Ngn1	B	E, A	BrdU, Mash1	D^{rt}	Cau *et al.*, 1997, 2002; Manglapus *et al.*, 2004
		Ngn2	N^{r}	E			Cau *et al.*, 1997
		Olig2	B?, I	E	β-Tubulin		Takebayashi *et al.*, 2000
	V	Id1	B?, Ap?	E			Jen *et al.*, 1996
		Id2	B, Ap^{r}	E, A			Jen *et al.*, 1996,Tietjen *et al.*, 2003
		Id3	B, I?, Ap?^{r}	E, A			Jen *et al.*, 1996; Tietjen *et al.*, 2003
		Id4	I?	E			Jen *et al.*, 1996
	VI	Hes1	Ap	E, A		D^{t}	Cau *et al.*, 2000, 2002; Manglapus *et al.*, 2004
		Hes5	B^{t}	E, A		D^{re}	Cau *et al.*, 2000, 2002; Manglapus *et al.*, 2004
		Hes6	B	E, P, A			Suzuki *et al.*, 2003a; Tietjen *et al.*, 2003
	NSCL	NSCL1	I^{t}	E, P			Suzuki *et al.*, 2003b

Table 1. Continued

Family	Class/group	TF	Expression profiles: In OE	Expression profiles: Age	Expression profiles: Co-expression	Function	References
		NSCL2	I^t	E, P			Suzuki *et al.*, 2003b
	O/E	Mmot1	N	E			Malgaretti *et al.*, 1997
		O/E-1	B, I	E, P, A	NCAM		Behrens *et al.*, 2000; Cau *et al.*, 2002; Davis and Reed, 1996; Wang *et al.*, 1997, 2002, 2004
		O/E-2	B, I	E, P, A		$Innervation^r$	Wang *et al.*, 1997, 2002, 2004
		O/E-3	B, I	E, P, A	β-Tubulin	In^r	Wang *et al.*, 1997, 2002, 2004
		O/E-4	B, I	E, A			Wang *et al.*, 2002
HD	Dlx	Dlx5	N	E, P		D^t, In	Depew *et al.*, 1999; Levi *et al.*, 2003
	Emx	Emx1	N*	E, P, A			Briata *et al.*, 1996, Nédélec *et al.*, 2004
		Emx2	B, I*	E, A	OMP	In	Yoshida *et al.*, 1997; Mallamaci *et al.*, 1998; Nédélec *et al.*, 2004
	LIM	Lhx2	B, I	E, A	β-Tubulin, BrdU	D^r	Cau *et al.*, 2002; Hirota and Mombaerts, 2004; Kolterud *et al.*, 2004
	Msx	Msx1	B^r	A			Norlin *et al.*, 2001
	Otx	Otx1	N	E			Simeone *et al.*, 1993
		Otx2	N	E			Simeone *et al.*, 1993; Mallamaci *et al.*, 1996
	Paired	Pax6	B, I, Ap^t	E, P, A			Davis and Reed, 1996; Behrens *et al.*, 2000
		Phd1	B, I	E, P	BrdU		Cau *et al.*, 2002; Saito *et al.*, 1996
	POU	Brn3a	N	E			Wagner *et al.*, 2005

Table 1. Continued

Family	Class/group	TF	Expression profiles: In OE	Expression profiles: Age	Expression profiles: Co-expression	Function	References
	Six	Six1	B, Ap	E, A			Tietjen *et al.*, 2003
MBD		Mecp2	B, I, Ap^t	E, P, A	β-Tubulin, OMP	D^t, In(g)	Cohen *et al.*, 2003; Matarazzo *et al.*, 2004
NFI		NFI-A	I, Ap^t	E, P			Behrens *et al.*, 2000
		NFI-B	Ap^t	E, P			Behrens *et al.*, 2000
		NFI-C	B, I, Ap^t	E, P			Behrens *et al.*, 2000
		NFI-X	N^t	E, P			Behrens *et al.*, 2000
Runx		Runx1	B	E, P	β-Tubulin, Ki67, Mash1, NeuroD	D	Theriault *et al.*, 2005
Sox		Sox2	N	E			Cau *et al.*, 1997
		Sox11	B	E,A			Tietjen *et al.*, 2003
TEA		ETF	B	E,A			Tietjen *et al.*, 2003
Zn finger	C_2H_2	CTIP2	N	E			Leid *et al.*, 2004
		KLF7	N	E, P		D?, In	Laub *et al.*, 2001, 2005
		Roaz	B, I	A			Tsai and Reed, 1997
		Wt1	B, I?	E	Mash1, NCAM	D	Wagner *et al.*, 2005

Note. A—adult; Ap—apical; Atf—activating TF; B—basal; BF—1-brain factor 1; BrdU—bromodeoxyuridine; Brn3a— Brain 3a; CTIP—chicken ovalbumin upstream promoter TF—interacting protein**;** D-development; E—embryonic; Emx—vertebrate homeobox gene related to *empty spiracles* (*ems*); ETF—embryonic TEA domain containing TF; Fox—Forkhead box; (g)—glomeruli; HD—homeodomain; Hes—mammalian homolog of *hairy* and *Enhancer of split*; HLH—helix—loop–helix; I—intermediate; Id—inhibitor of DNA binding; In—innervation; Ki67—mitotic cell-marker protein; KLF7—Krüppel-like factor 7; Mash1—mammalian *achaete-scute* homolog 1; MBD—methylated-CpG binding domain; Mecp2—methyl-CpG binding protein 2; Mmot1—metencephalon-mesencephalon-olfactory TF 1; N—not specified; NCAM—neural cell adhesion molecule; NFI— nuclear factor I; Ngn—neurogenin; NSCL—neural stem cell leukemia; O/E—Olf (olfactory neuronal TF)/Ebf (early B cell TF); OMP—olfactory marker protein; Otx—vertebrate homeobox gene related to *orthodenticle* (*otd*); P—postnatal; Phd1—paired homeodomain 1; r—region specific; re—redundant function; Roaz: rat O/E—1-associated zinc finger; Six—vertebrate homolog of *sine oculis*; *t*—time dependent; Wt—Wilms' tumour gene; Zn—zinc; *—axonal expression seen.

contrast, GBCs, which are primarily situated immediately apical to the HBCs (Fig. 1(A)), are morphologically round cells (Graziadei and Monti Graziadei, 1979; Holbrook *et al.*, 1995).

With the aide of lineage tracers, such as replication-incompetent retroviruses, researchers have identified cells in the basal region of the OE that appear to give rise to neuronal and non-neuronal cells in rats exposed to methyl bromide (Schwob *et al.*, 1994). Whether these cells are GBCs or HBCs is still debatable, since both cell types display increased BrdU expression in response to methyl bromide exposure (Schwob *et al.*, 1995). Interestingly, a recent study suggests that HBCs may be the multipotent progenitor cells of the OE since they display characteristics exhibited by stem cells in other regions of the body (Carter *et al.*, 2004). However, further research is needed to substantiate this finding.

Similar studies utilizing tritiated thymidine and replication-incompetent retroviruses in normal, bulbectomized, or transected mice have also identified cells in the basal region of the OE that appear to give rise to neurons. Based on their morphological and antigenic phenotype these cells appear to be GBCs (Schwartz Levey *et al.*, 1991; Caggiano *et al.*, 1994; Schwob *et al.*, 1994). Interestingly, researchers have found through tissue culture experiments that GBCs appear to be a heterogeneous population of cells consisting of early transit amplifying cells and late immediate neuronal precursors (INPs) (Calof and Chikaraishi, 1989; Gordon *et al.*, 1995). Transit amplifying cells, which are believed to be the direct progeny of stem cells, comprise an intermediate population of committed progenitors that have a lower capacity for self-renewal than do stem cells but a higher probability of initiating terminal differentiation after several cell divisions. Thus, transit amplifying cells serve to increase the number of differentiated cells produced by each stem cell division. In the olfactory epithelium they are involved in tissue repair after injury as well as playing a role in the regular renewal of OSNs.

Following one or more divisions, the INPs exit the cell cycle and differentiate into immature OSNs, which are located immediately apical to the GBCs in the intermediate region of the OE (Graziadei and Monti Graziadei, 1979; Calof and Chikaraishi, 1989). Immature OSNs are bipolar cells that express β-tubulin, GAP43, SCG10, and NCAM (Fig. 1(A)) (Calof and Chikaraishi, 1989; Roskams *et al.*, 1998; Verhaagen *et al.*, 1989; Pellier-Monnin *et al.*, 2001). Development of these cells into mature OSNs involves the elaboration of a dendrite and axon, which project to the apical or basal surfaces, respectively. The dendrite is characterized by the presence of a dendritic knob, which extends beyond the apical surface of the OE and bears numerous cilia (de Lorenzo, 1957; Frisch, 1967). The cilia express many proteins that are important in odorant signal transduction, including G_{olf}, adenylyl cyclase III (ACIII), olfactory cyclic nucleotide-gated channel (OcNc), and odorant receptors (ORs) (Jones and Reed, 1989; Bakalyar and Reed, 1990; Brunet *et al.*, 1996; Belluscio *et al.*, 1998; Matsuzaki *et al.*, 1999; Wong *et al.*, 2000; reviewed in Ronnett and Moon, 2002). Interestingly, each murine OSN expresses only one of approximately 1000 OR genes (Malnic *et al.*, 1999; Zhang and Firestein, 2002) which has led to the suggestion that it is the unique combination of OSNs responding to each odor that allows for the discrimination of a wide variety of odors (Malnic *et al.*, 1999).

The OSN axons upon exiting the basal side of the OE fasciculate and traverse the cribiform plate to form the outer layer of the OBs. Upon reaching the OBs the axons defasciculate and target different regions (Whitesides and LaMantia, 1996), terminating in structures called glomeruli (Graziadei and Monti Graziadei, 1979). Interestingly, all sensory neurons terminating on a particular glomerulus have been shown to express the same odorant receptor (OR) (Mombaerts *et al.*, 1996).

TRANSCRIPTIONAL REGULATION: OSN PROGENITORS

Neurogenesis in the OE has been very well characterized in terms of the phenotype of cells as they progress through the lineage (Fig. 1(A)). Thus, the expression profile of a particular TF can be very useful in predicting its role in OSN development. For example, since Mash1 is expressed in apical, intermediate, and basal regions of the murine embryonic OE (Cau *et al.*, 1997; 2002; Theriault *et al.*, 2005), it could function at various stages of OSN development. However, as it is expressed by mitotic transit amplifying progenitor cells and not differentiated OSNs (Fig. 1(A)) (Gordon *et al.*, 1995; Cau *et al.*, 1997), it must act early in the OSN lineage. Indeed, in the absence of Mash1 there is a drastic reduction in mitotic basal progenitors, as well as, in β-tubulin$^+$ and SCG10$^+$ neurons in the OE (Table 2) (Guillemot *et al.*, 1993; Cau *et al.*, 1997; 2002). This, in turn, corresponds with a dramatic increase in the expression of *Steel*, which marks sustentacular cells, in the mutant OE (Murray *et al.*, 2003). Therefore, these findings suggest that Mash1 could be critical for neuronal determination in the OE. As Mash1 is not expressed in the ventrocaudal region of the OE, it is not surprising that OSN development proceeds normally in this region in the absence of Mash1 (Cau *et al.*, 1997).

Two TFs that have been shown to affect Mash1 expression in the olfactory placode or OE during embryonic development are Hes1 (mammalian homolog of *Drosophila Hairy* and *Enhancer of split*; Cau *et al.*, 2000) and Wt1(+KTS) (Wilms' tumor zinc-finger protein; Wagner *et al.*, 2005) (Fig. 1(B) and Table 2). Hes1 is expressed on the apical side of the embryonic OE (Table 1) (Cau *et al.*, 2000; 2002), which contains a large number of mitotic cells (Smart, 1971). Therefore, based on its expression profile Hes1 could play a role very early in OSN development. However, Hes1 is a downstream effector in the Notch signaling pathway (Nishimura *et al.*, 1998), which is dependent on Mash1 for the correct expression of the Notch ligands (Cau *et al.*, 2002). Therefore, Hes1 would be expected to act downstream of Mash1. Surprisingly, although Hes1 expression is drastically downregulated at E12.5 in the Mash1 murine mutant OE, its expression at E10.5 is largely unaffected by the absence of Mash1 (Cau *et al.*, 2000, 2002). This suggests that Hes1 plays an early role independent of Mash1 expression. Indeed, Hes1 appears to be a limiting factor of neurogenesis since there is a dramatic increase in the number of Mash1$^+$ cells at E10.5 in its absence. In addition, there is a significant increase in the number of SCG10$^+$ OSNs at E12.5 (Table 2) (Cau *et al.*, 2000). The delayed increase in neuronal numbers may be due to the presence of an additional Hes TF, Hes5, which is also a downstream effector of the Notch-signaling pathway (Nishimura *et al.*, 1998)

Table 2. Transcriptional Control of OSN Development Elucidated With the Aide of Transgenic Knockout Mice

	Cell dynamics		OSN marker expression			TF expression			
TFmutant	↓	↑	↓/Ø	↑	↔	↓/Ø	↑	↔	References
Dlx5			β-tubulin^{r} NCAM^{r} OMP^{rt}						Levi *et al.*, 2003; Long *et al.*, 2003
Hes1				SCG10^{t}			Hes5 Mash1 Ngn1		Cau *et al.*, 2000
Hes5					SCG10			Mash1 Ngn1	Cau *et al.*, 2000
KLF7			NCAM						Laub *et al.*, 2005
Lhx2		Cell death	G_{olf} OMP^{r} ORs*				NeuroD^{r}	Mash1 Msx1 Ngn1	Hirota and Mombaerts, 2004; Kolterud *et al.*, 2004
Mash1	Mitosis^{r}	Cell death^{t}	β-tubulin^{rt} OMP^{r} NCAM^{r} SCG10^{rt}			Hes1^{rt} Hes5^{r} Hes6 Lhx2^{r} NeuroD^{r} Ngn1^{r} O/ E1^{r} Phd1^{r}		ETF Ngn2 Pax6 Six1 Sox2 Sox11	Cau *et al.*, 1997; 2000, 2002; Guillemot *et al.*, 1993; Saito *et al.*, 1996; Murray *et al.*, 2003; Tietjen *et al.*, 2003
Mecp2	Cell death^{t}	Mitosis^{t}	BrdU/OMP ^{t}	BrdU/ GAP43^{t}				O/E-1	Matarazzo *et al.*, 2004
Ngn1			SCG10^{rt}			Lhx2 Neuro D O/E1 Phd1		Hes1 Hes5 Mash1	Cau *et al.*, 2002
O/E-2					OMP				Wang *et al.*, 2004
O/E-3					M4 M72 O MP OcNc2				Wang *et al.*, 2004
Runx1	Mitosis			β-Tubulin		NeuroD		Mash1	Theriault *et al.*, 2005
Wt1 (+KTS)	Mitosis	Cell death				Brn3a Mash1 Ngn1			Wagner *et al.*, 2005

Note. r—region specific; t—time dependent; ↓—decreased; ↑—increased; Ø—absent; ↔—unchanged; *—ORs shown to be affected include A16, K21, L45, M12, M50, M71, M72, MOR23, MOR251-4, and P2.

and, hence, could prevent premature differentiation in the absence of Hes1. Indeed, Hes1/Hes5 double mutant mice exhibit a large increase in the number of SCG10$^+$ neurons at E10.5 (Cau *et al*., 2000). Cau *et al.* (2000) proposed a model in which Hes1 initially limits the extent of neurogenesis in the embryonic OE, whereas both Hes1 and Hes5 function synergistically to prevent premature differentiation of OSNs by acting as downstream effectors in the Notch signaling pathway (Fig. 1(B)). Whether Hes1 and Hes5 play similar roles in the adult OE remains to be determined.

Unlike Hes1, Wt1 is expressed predominantly in the basal region of the embryonic OE (Table 1). Wt1 and Mash1 have been shown to be co-expressed in a proportion of cells in the OE (Wagner *et al*., 2005). There are several lines of evidence that support the role of Wt1 as an upstream effector of Mash1. Firstly, selective ablation of the alternatively spliced Wt1(+KTS) isoform results in a dramatic decrease in the expression of Mash1 in the developing OE (Wagner *et al*., 2005). Transfection of HEK293 cells with Wt1(+KTS) results in an upregulation of Mash1 mRNA and protein expression. However, co-transfection of HEK293 cells with a Mash1 promoter construct and Wt1(+KTS) is unable to significantly enhance the activity of the Mash1 promoter even though it contains two predicted Wt1(+KTS) binding sites (Wagner *et al*., 2005). Hence, further research is needed to determine if additional factors are required for Wt1(+KTS) to activate Mash1 expression or whether Wt1(+KTS) could act post-transcriptionally, as suggested by Wagner *et al.* (2005). In addition, analysis of Mash1 expression in Wt1(+KTS) wild-type and mutant mice at earlier stages of embryonic development may determine whether Wt1(+KTS) truly induces Mash1 expression or merely maintains it.

TRANSCRIPTIONAL REGULATION: OSN DIFFERENTIATION

Multiple TFs have been suggested or shown to act downstream of Mash1, including Runx1 and Ngn1, respectively (Table 2) (Cau *et al*., 2002; Theriault *et al*., 2005). Runx1 appears to be expressed predominantly in the basal region of the OE where it exhibits overlapping expression with Mash1 and NeuroD. In addition, a small number of Runx1-expressing cells appear to co-express β-tubulin (Table 1). Runx1 appears to act downstream of Mash1 since Mash1 expression is unaltered in Runx1-deficient mice (Table 2) (Theriault *et al*., 2005). In fact, Runx1 is important in preventing premature differentiation of OSN progenitors since Runx1-deficient mice exhibit a significant decrease in the number of NeuroD$^+$ cells, as well as, an increased number of β-tubulin$^+$ OSNs (Table 2). How Runx1 prevents premature differentiation is unknown but may involve its ability to repress the expression of the cyclin dependent kinase inhibitor, p21 (Theriault *et al*., 2005), which is involved in cell cycle exit (Luo *et al*., 1995).

As early OSN progenitors are present in Runx1-deficient mice (Theriault *et al*., 2005), other TFs must compensate for Runx1 in its deficiency. Possible candidates include Atf5 and Id4 TFs, which have been shown to inhibit neuronal differentiation in the brain (Lyden *et al*., 1999; Angelastro *et al*., 2003) and are expressed in the OE (Table 1) (Jen *et al*., 1996; Hansen *et al*., 2002). Unlike the brain, however,

Atf5 and Id4 do not appear to be expressed by progenitor cells (Jen *et al*., 1996; Hansen *et al*., 2002; Angelastro *et al*., 2003) and, therefore, may have divergent roles depending upon the tissue in which they are expressed. Thus, further research is needed to decipher the role of Atf5, Id4 and other TFs in the correct timing of OSN differentiation.

Although Ngn1 is expressed by basal progenitor cells, some of which also express Mash1 (Fig. 1(A) and Table 1), it likely plays a role in OSN differentiation since it acts downstream of Mash1 in most of the OE (Cau *et al*., 1997; 2002). Indeed, there is a delay and a transient dramatic decrease in SCG10 expression in the absence of Ngn1 (Table 2), which is more severe in the caudal region of the mutant OE (Cau *et al*., 2002). Mash1 is not normally expressed in the ventrocaudal region of the OE (Cau *et al*., 1997), so Ngn1 may have an earlier function in this region. In support of this, SCG10 expression is absent in the embryonic OE of Mash1/Ngn1 double mutant mice (Cau *et al.*, 2002). In addition, another Ngn TF, namely Ngn2, is also expressed in the ventrocaudal region of the OE (Cau *et al*., 1997) and hence could account for the transient nature of the Ngn1 mutation in this region. Since Ngn2 expression is limited to the ventrocaudal region (Cau *et al*., 1997), additional TFs must compensate for Ngn1 in other regions of the OE. One potential candidate is the HLH TF Olig2, which is expressed by OSNs (Table 1) (Takebayashi *et al*., 2000) and has been shown to be important in motor neuron development in the spinal cord (Lu *et al*., 2002).

As Ngn1 has been shown to regulate the expression of Hes6 and NeuroD (Koyano-Nakagawa *et al*., 2000; Cau *et al*., 2002), OSN differentiation may also be regulated by these TFs. Although their roles in OSN differentiation have not been elucidated, evidence exists in other neuronal lineages in support of this. In particular, researchers have found that overexpression of NeuroD in E20 rat retinal explants results in premature differentiation of late-born retinal neurons (Ahmad *et al*., 1998). Similarly, Hes6 has been shown to promote neuronal differentiation in the retina and neural plate (Bae *et al*., 2000; Koyano-Nakagawa *et al*., 2000). As both of these TFs exhibit overlapping expression profiles with Ngn1 in the OE (Cau *et al*., 1997; Suzuki *et al*., 2003a), they are ideally positioned to act downstream of Ngn1 in promoting OSN differentiation (Fig. 1(B)). Interestingly, Hes6 has been shown to abolish Hes1-induced transcriptional repression (Bae *et al*., 2000). Koyano-Nakagawa *et al*. (2000) have proposed that one way in which Hes6 may promote neuronal differentiation is by means of a positive feedback loop in which Hes6 inhibits the expression/activity of HLH repressors (e.g. Hes1; Bae *et al*., 2000) and therefore indirectly enhances Ngn1 expression.

Although the OE expression of the LIM homeobox TF, Lhx2, appears to be only slightly reduced in Ngn1 mutant mice, it is strongly reduced in the absence of Mash1 (Cau *et al*., 2002). Therefore, Lhx2 must act downstream of Mash1 in the regulation of neurogenesis in the OE. As Lhx2 is expressed in the basal and intermediate regions of the OE (Cau *et al*., 2002; Hirota and Mombaerts, 2004; Kolterud *et al*., 2004), it may play a role in OSN differentiation (Table 1). Numerous OR genes, which are expressed by immature and mature OSNs (Iwema and Schwob, 2003), are absent in Lhx2 mutant mice (Hirota and Mombaerts, 2004; Kolterud *et al*.,

2004). Unfortunately, it is difficult to identify the exact function of Lhx2 due to discrepancies in reported effects on OSN differentiation in mutant mice. In particular, Hirota and Mombaerts (2004) found that SCG10, GAP43 and OMP expression is reduced, severely reduced or absent, respectively, in Lhx2 mutant mice. In contrast, Kolterud *et al.* (2004) found expression of SCG10, GAP43 and OMP in the Lhx2 mutant OE. As both groups utilized the same mutant mouse line, these discrepancies cannot be explained by different mutant phenotypes. Instead, the discrepancies may be due to selection of different regions of the OE for study since Kolterud *et al.* (2004) found OMP^+ OSNs were selectively situated in the dorsomedial region of the nasal cavity of the mutant OE mice. Interestingly, both research groups found that the Lhx2 mutant mice exhibit an increase in the number of $NeuroD^+$ cells in the OE (Table 2) (Hirota and Mombaerts, 2004; Kolterud *et al.*, 2004). As NeuroD is expressed by mitotic cells, as well as, some β-$tubulin^+$ OSNs (Cau *et al.*, 1997), Lhx2 could either inhibit the progression of neurogenesis at the level of the progenitor or early differentiated neuron. Current evidence supports the latter, since there is an increased number of $NeuroD^+/\beta$-$tubulin^+$ cells in the Lhx2 mutant OE (Kolterud *et al.*, 2004). Further research is needed to decipher the role of Lhx2 in OSN differentiation.

TRANSCRIPTIONAL REGULATION: OSN MATURATION

There are a number of TFs that are not critical for OSN differentiation but instead appear to be important in OSN maturation and function. For example, Olf-1 binding sites, to which all O/E TFs have been shown to bind (Malgaretti *et al.*, 1997; Wang *et al.*, 1997; 2002), have been identified in the promoter regions of several markers expressed by mature OSNs including OMP, G_{olf}, OcNc, and ACIII (Kudrycki *et al.*, 1993; Wang *et al.*, 1993). Furthermore, co-transfection experiments have shown that O/E TFs can induce reporter gene expression driven by the OMP and ACIII promoters (Wang *et al.*, 1997).

As O/E TFs are expressed in basal and intermediate regions of the OE (Table 1) (Wang *et al.*, 1997; 2002; Cau *et al.*, 2002), which contain progenitors and OSNs, respectively, additional factors must interfere with their ability to enhance OMP, G_{olf}, OcNc and/or ACIII expression at early stages of OSN development. In fact, there appear to be numerous factors that could determine their functional activity. For example, a zinc finger protein, Roaz, interacts with O/E TFs and inhibits their transactivation of OSN specific genes in vitro (Tsai and Reed, 1997). As Roaz exhibits an overlapping expression profile with the O/E TFs in the basal and lower intermediate regions of the OE (Tsai and Reed, 1997; Wang *et al.*, 1997; 2002), it may inhibit their function during early stages of OSN development. In addition, NFI TFs, which also have binding sites in the promoters of OMP, OcNc and ACIII (Baumeister *et al.*, 1999), appear to interfere with O/E activity since co-transfection of HEK293 cells with O/E-1 and an OMP promoter construct containing a mutated NFI binding site results in an increase in the activation of the OMP promoter by O/E-1 (Behrens *et al.*, 2000). As NFI-A and NFI-C appear to exhibit overlapping

expression domains with the O/E TFs (Table 1) (Behrens *et al.*, 2000; Wang *et al.*, 1997; 2002), they could interfere with O/E function in vivo. Furthermore, the TF O/E-4 appears to be a much weaker transcriptional activator than the other members of the O/E family (Wang *et al.*, 2002) and, therefore, may interfere with their function.

OMP expression is, however, detected in O/E-1-, O/E-2-, and O/E-3-deficient mice (Wang *et al.*, 2004; Lin and Grosschedl, 1995). Although other family members may compensate for these O/E TFs in their absence, the findings of Kudrycki *et al.* (1998) suggest this is not the case since reporter gene expression is seen in the OSNs of transgenic mice in which the OMP reporter construct contains a mutated Olf-1 binding site. As a result additional TFs may play a more important role than O/E TFs in regulating OMP expression. Alternatively, O/E TFs may be more critical for the expression of G_{olf}, ACIII and/or OcNc. Hence, further research is needed to determine if O/E TFs regulate the expression of G_{olf}, ACIII, and/or OcNc, as well as, whether olfaction is impaired in these mutant mice.

Interestingly, olfactory axons fail to innervate the lateral and/or dorsal regions of the OBs in O/E-2 and O/E-3 deficient mice (Wang *et al.*, 2004). As O/E-2 and O/E-3 are expressed in the OE, but not in the nasal mesenchyme or the OB, the aberrant innervation of target tissue by olfactory axons is most likely a direct effect of the absence of O/E-2 and O/E-3. Hence, further research is needed to clarify the role of O/E TFs in the maturation of OSNs and in the ability of their axons to innervate the OB.

One TF that appears to be important in the maturation of OSNs is the methylated-CpG binding domain protein Mecp2. As the majority of $Mecp2^+$ cells co-express OMP in the postnatal OE with very few co-expressing β-tubulin (Cohen *et al.*, 2003), it is ideally positioned to act in OSN maturation. Interestingly, Rett's syndrome patients, who have been shown to exhibit mutations in the *MECP2* gene (Amir *et al.*, 1999; Lee *et al.*, 2001), exhibit an increased ratio of β-tubulin to OMP expression in OE biopsy specimens (Ronnett *et al.*, 2003) as well there is an impairment of OSN maturation in the OE of Rett's Syndrome patients (Ronnett *et al.*, 2003). Similarly, Mecp2 mutant mice exhibit a transient delay in the transition of OSNs from immature $GAP43^+$ cells to mature OMP^+ cells. These mutant mice also exhibit transient abnormalities in olfactory axon projections in the glomeruli (Matarazzo *et al.*, 2004).

Interestingly, Mecp2 has been shown to be involved in the imprinting of homeobox TF Dlx5 (Horike *et al.*, 2005), which is also expressed in the OE (Depew *et al.*, 1999; Levi *et al.*, 2003). In particular, Mecp2 mutants exhibit an increase in Dlx5 expression, which appears to be due to an increase in the paternal allele expression (Horike *et al.*, 2005). Although the effects seen in Mecp2 mutant mice could be due to an increased Dlx5 expression, there are several findings that do not support this. There is a transient delay in the onset of OMP expression in the absence of Dlx5 (Levi *et al.*, 2003) which is accompanied by the disruption of OB axonal innervation in these mutant mice (Levi *et al.*, 2003; Long *et al.*, 2003). One complicating factor in determining Dlx5's role in OSN maturation and its potential connection to Mecp2 is that Dlx5 mutant mice exhibit abnormalities in the OBs, as well as, the nasal

mesenchyme that could indirectly affect neurogenesis in the OE (Depew *et al.*, 1999; Levi *et al.*, 2003; Long *et al.*, 2003). A further complication is the fact that there are two Dlx5 mutant mouse strains that vary in the severity of olfactory placode/OE anomalies (Depew *et al.*, 1999; Levi *et al.*, 2003; Long *et al.*, 2003). Hence, further research is needed to decipher the role of Dlx5, as well as, its regulation by Mecp2 in OSN development.

Another TF that appears to be important in OSN maturation is the Kruppel-like TF KLF7 (Laub *et al.*, 2001). Although there are no significant changes in OE thickness, cell density, proliferation, or apoptosis in KLF7 mutant mice, there is a reduced number of $NCAM^+$ OSNs that also exhibit reduced axon growth in these mutant mice at E11.5. Subsequently, olfactory axons fail to innervate the OBs at later stages of development in KLF7 mutant mice. Interestingly, the mutant OE exhibits a dramatic decrease in the expression of p21, which has been shown to promote cell cycle exit, as well as, neurite outgrowth (Luo *et al.*, 1995; Tanaka *et al.*, 2002). Hence, further research is needed to determine whether the mutant's anomalies are due to reduced p21 expression or whether other factors are involved.

CONCLUSION

Over the past decade there have been significant advances in our understanding of the transcriptional control of neurogenesis in the OE. Although numerous TFs are expressed in regions of the OE containing cells of the OSN lineage, thus far only a handful have been identified with the aide of transgenic knockout mice as playing roles in OE neurogenesis (Table 1). Mash1 has emerged as an important proneural gene in olfactory neurogenesis, with the transient expression of this TF by the transit amplifying GBCs appearing to be a critical step in their progression to a neuronal fate (Guillemot *et al.*, 1993; Gordon *et al.*, 1995; Cau *et al.*, 1997; 2002; Murray *et al.*, 2003). Accordingly TFs shown to regulate Mash1 expression, such as Hes1 and Wt1(+KTS), exhibit abnormalities in OE neurogenesis. Numerous TFs have also been identified that act downstream of Mash1, including Ngn1, NeuroD, Hes TFs, and Lhx2 (Fig. 1(B)) that appear to be important in OSN differentiation. In addition, a handful of TFs, such as Mecp2, Dlx5, and KLF7, appear to be important in OSN maturation and functional innervation of the OBs.

Unfortunately, there are limitations to the usefulness of transgenic knockout mice in elucidating the function of TFs in OE neurogenesis. For example, in some cases multiple members of the same TF family, such as the O/E TFs, are expressed by OSNs (Table 1) and, hence, could play redundant roles. As a result, a mutation in only one member of the TF family may not uncover function due to the compensatory effects of the other members. In addition, for TFs that play key roles very early in embryonic development the ability to determine their role in later developmental events such as OSN differentiation or maturation may be severely hampered when using the transgenic knockout approach. For example, Pax6 and Otx2 are important in patterning the head since in their absence several sensory structures, including the olfactory placodes, are absent (Acampora *et al.*, 1995; Grindley

et al., 1995). Thus, it remains to be determined if their effects on olfactory placode development are direct or whether they arise as a consequence of abnormal patterning of a number of structures in the head. Thus experimental models involving gain-of-function, as well as, conditional or multiple gene ablations may be required to determine the function of some of the TFs in OE neurogenesis.

In addition, there are many aspects of the regulation of OE neurogenesis that remain to be elucidated. For instance, a clear sense of how the transcriptional cascade (Fig. 1(B)) interacts with the positive and negative microenvironmental signals (Calof *et al*., 2002, 1996; Schwob, 2002) that regulate the state of dynamic equilibrium of neurogenesis in the OE is lacking. Furthermore, there is a major gap in our knowledge of OE neurogenesis in regards to the transcriptional control of OR gene selection by individual OSNs. Researchers have found that OSNs expressing the same OR are randomly distributed in one of four zones in the OE (Sullivan *et al*., 1995). The only TFs that have been shown to exhibit expression patterns reflective of a particular OR zone(s) are Msx1 (Norlin *et al*., 2001), Id2, and Id3 (Tietjen *et al*., 2003); however, whether they are important for OR expression is currently unknown. Finally, the transcriptional control of neurogenesis in the human OE needs to be examined in order to determine if similarities and differences exist between rodents and humans. Recent research has shown that many similarities, as well as, differences are seen in the cellular architecture of human and rodent OE (Hahn *et al.*, 2005), which may be reflected in their regulation.

The importance of elucidating the transcriptional control of OE neurogenesis is that it has the potential of providing a tool with which to explore the pathophysiology of developmental and age-related diseases in humans. In particular, findings in laboratory animals can be readily tested in human biopsy tissue obtained from donors as young as 2 years of age (Ronnett *et al*., 2003) when the pathophysiology of Rett's Syndrome, for example, is actively progressing, or as old as elderly Alzheimer's disease patients to study the pathophysiology of age-related diseases. It also provides an opportunity to study single-cell transcriptomes in neuronal progenitor cells and in mature neurons obtained from an easily accessible human tissue source (Tietjen *et al.*, 2003).

ACKNOWLEDGMENTS

Supported by a research grant from the Canadian Institutes of Health Research (CIHR) and the Multiple Sclerosis Society of Canada to AJN and JRD. DJN is supported by a CIHR Health Research Fellowship

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